

Will there ever be peace in Europe?

Settling Yugoslavia's tribal wars is an urgent need, but in the long run Europe must trade cultural diversity for economic development away from its agrarian roots.

SINCE the time of Charlemagne, Europe has been a turbulent place. The only comfort is that it was even worse before: the Roman Empire was never as stable and prosperous as its contemporary apologists would have had the rest of us believe. The swashbucklers who built that empire took it as their first task in newly occupied parts of Europe to pacify the warring tribes that had previously been in charge; Tacitus tells the tale well in respect of Britain. Rarely did they succeed entirely. Indeed, the post-Roman history of Europe has been one of continuous tribal conflict, complicated by the frequent invention of new tribes (such as the Normans, itinerant warlords with Scandinavian origins) and moderated occasionally by the temporary emergence of central authority — the Holy Roman Empire, the Napoleonic regime and the Stalinist recipe that kept the tribes of Central Europe at peace for 70 years in some cases, and since 1945 in the swathe of land from the Baltic to the Mediterranean, in the Adriatic and Aegean.

So what can hold the old tribal rivalries in check now that the stabilizing recipe has been abandoned and discredited? Events of the past few months in Yugoslavia point to the need for cement of another kind. But hankerings after the late Marshal Tito's recipe for stability would serve no purpose. The tragic warfare of the past several months, first in Croatia and now in Bosnia, shows that Tito's illusion of stability did nothing to blunt the edge of tribal rivalry in the Balkans but instead, by suppression, may have aggravated it. But the Serbian (and also Croatian) euphemism for justifying land-grabbing that turns peaceable people into refugees — 'ethnic cleansing' is the phrase — is such a painful echo of other recent abominations, by Stalin as well as Hitler, that the rest of Europe should be thoroughly alarmed, perhaps even in arms.

Yet much of Europe is indifferent to what is happening in ex-Yugoslavia. To be sure, the United Nations peace-keeping force, largely Canadian by nationality but with significant French support, is now trying to police in Bosnia, as it tried in Croatia, cease-fire agreements that are broken as soon as they are supposed to operate. There are also negotiations in prospect, an outcome of the European Communities' (EC's) uncertain initiative, whose chief outcome so far has been Lord Carrington's long tally of broken cease-fire agreements. But Germany, which helped to precipitate the trouble by its over-hasty recognition of Slovenia last December, is unlikely to win much response to its plea for help with refugees, of whom it has taken

200,000 so far. (The irony is that in the long run it may not matter much; if granted asylum under German law, refugees from Yugoslavia will be free to live wherever they can find jobs in the EC.)

What the rest of Europe must now do is to settle on a policy on Yugoslavia if a brokered peace, the best solution, does not emerge from the current London talks. The starting point should be a recognition that even governments have no right to turn people out of their homes because their language or their religion does not match their neighbour's, and that these despicable practices, by Croats as well as Serbs, are an offence against European civility that cannot be hidden by the pretence that either Yugoslavia or its components are sovereign states. Military intervention would probably be ineffectual, but is probably not necessary. Europe has not yet tried the concerted political pressure it could exert, and the effective sanctions that could follow. It would help enormously to engender sobriety in Bosnia if there were a public dossier of those known to be responsible for atrocities against the time when parts of Yugoslavia will wish to rejoin the wider European community.

That is a short-term strategy. How is Europe to bury the more durable tribal rivalries represented by Ulster, the continuing trouble over the Basque region of Spain and the impending secession of Slovakia from Bohemia and Moravia? For all of Europe's boast that it embodies a continental culture, too much of it is trapped in an agrarian way of life, which makes for an immobile population and the exaggeration of linguistic and other cultural differences. Thus the long-term remedy must be economic development away from agriculture — Europe can overfeed itself easily — and a wider economic union, which is the case for enlargement of the EC. But would that not diminish the cultural diversity of Europe? Those who make that argument an absolute should ask people who live in Bosnia what diversity has done for them.

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Welcoming Wellcome

Not every day (worse luck) is the equivalent of a new research council magicked into being.

BRITAIN'S Medical Research Council (MRC) has a distinguished reputation, most conspicuously for having supported molecular biology in its early days. MRC, of course, remains the mainstay of medical research, and of much of British biology as well. So would it not be a day of great rejoicing for British research if there were a second research council on the same scale and with similar objectives? That may be what happened last week, when the Wellcome Trust, originally founded as the charitable arm of a pharmaceutical manufacturer (called the Wellcome Foundation in Britain) cleverly used the City of London to double its annual income to more than £200 million. Indeed, the prospect of such a jump in spending on medical and biomedical research may be even better than the creation of a new research council, for foundations can, if they play their cards well, carry extra clout.

Of necessity, public research organizations such as MRC acquire unshakeable commitments, to people and to institutions, as the years go by. Although MRC's traditional policy is that research units and even institutes do not necessarily outlast their founders, it is not always easy to follow that recipe. And, indeed, it would make very little sense that international institutions such as the Laboratory of Molecular Biology at Cambridge should be closed in slavish pursuit of such a policy. But that means that increasing proportions of the funds available are tied up in fixed enterprises. Foundations, by contrast, can and should avoid fixed commitments. Their value, and their leverage, stems from the influence they can exert at the margins of research. The trick is to start good enterprises and, if necessary, to persuade even deeper pockets to take them over.

So what, in contemporary Britain, should the Wellcome Trust attempt? The plan to set up Dr John Sulston's group, now hard at work on the nucleotide sequence of the nematode, in a laboratory of its own is an excellent scheme, but responsibility for what happens when the first five years are up should rest as much with Sulston as with Wellcome. Even so, this and similar initiatives could do much to change the tone of British research. A few examples of well-founded laboratories whose occupants could demonstrate their freedom from the grinding impoverishment of the past decade or so could marvelously revive expectations in laboratories elsewhere. Is there any better way of helping younger people to make a decisive start on careers in research, of which there has been some talk since last week's announcement of the trust's sale of £2.1 billion's worth of shares in the foundation?

Wellcome's influence could thus extend far beyond the fields of science specified in its trust deed. The

trust's financial acumen should also be an example to other charitable foundations, many of which are for too long trapped by sentiment into investments that yield moderate or even miserable returns. Too often, trustees take the view that loyalty to a founding benefactor requires them to stick with the investments in their original endowment. Britain's Nuffield Foundation, the richest in Britain in the 1950s, would now be much more influential if it had not hung onto its shares in its founder's ailing motor manufacturer until the government bought them up for £0.10 each just twenty years ago. The moral is that foundations must be enterprising in looking after their endowments as well as in spending whatever income rolls in. □

Infinity denied

British Telecom, the privatized British telephone monopoly, is running out of numbers, but should not worry.

HARDLY anybody loves a telephone company, which is one reason why British Telecom has been persuaded by its regulator to postpone by (at least) a year the time when most of its subscribers would be required to add an extra digit to the numbers with which they (and their acquaintances) are familiar. But the whole fracas generated by British Telecom's announcement of its intentions is a failure of logic in the face of arithmetical reality. There are plenty of shorter numbers to go around.

Britain, in round numbers, has more than 10^7 telephone subscribers, but fewer than 10^8 . That means that eight-digit telephone numbers should be able to accommodate them all. But people trying to reach telephone numbers in London even from within the United Kingdom must now routinely dial ten digits. Rural locations are no more easily accessible. The mere suggestion that every British telephone number would be one digit longer quickly mobilized business groups to protest at the extra cost of printing new letterheads. Nobody seems to have bothered much about the waste of people's time entailed in dialling an extra digit for every telephone call.

The reason why British Telecom and telephone companies elsewhere are in such a muddle is that they persist in trying to endow telephone numbers with a meaning they cannot continue to enjoy. The standard illusion of telephone planners is that the first few digits of a telephone number have geographical significance — in the United States, for example, "415" means the Bay Area, or "212" New York. Second, they are trapped in the old electromechanical switching era when the digits in a telephone number were literally used sequentially as switching instructions, directing calls through one circuit or another according to their value. With stored program control now the norm in telephone exchanges, continuing efforts to associate the first few digits of a telephone number with the geographical location of the receiver (or fax machine) concerned are neither necessary nor prudent. □

Healy attacks NASA's claims; bad news for research budgets

Washington. The fierce competition for funds in the 1993 federal budgets now moving through the US Congress has shattered, possibly forever, the idea of a united front for science. The most visible sign of that splintering came last week when Bernadine Healy, director of the National Institutes of Health (NIH), rebuked Daniel Goldin, head of the National Aeronautics and Space Administration (NASA), for arguing that the proposed space station would be a boon to biomedical research. Her letter was dated one day before the US House of Representatives voted to spend \$1.73 billion next year on Space Station Freedom, and opponents of the space station cited it unsuccessfully during the debate before Goldin had even read it.

It is probably no coincidence that this internecine warfare between two officials of the Bush administration came at the same time as the House, in separate budget votes last week, warned NIH and the National Science Foundation (NSF) to prepare for the worst. For NIH, a budget that rises by 3 per cent not only falls short of the 5 per cent increase sought by the president but also ends a tradition in which Congress has added substantially to whatever the president requested. For NSF, a research budget that stays flat in 1993 dashes its request for a 13 per cent increase and makes a mockery of the promise by the president to double the foundation's budget in five years.

The House bill at least gives NIH \$297 million more than it now has, although the increase falls \$147 million short of the president's request and is less than is required to maintain the current level of new grants. Two days later, the Senate committee that oversees NSF's budget gave NSF some \$20 million less to spend on research in 1993 than in 1992, at the same time increasing spending for science edu-

cation by \$45 million and creating a \$55 million programme to retrain engineers now working on military projects. The levels for NSF are expected to be ratified by the entire Senate this week; however, action on the NIH budget is not scheduled until the middle of September. In each case, differences between the two houses must be ironed out in conference.

opportunities. And they do not want to be used as the justification for a project they oppose.

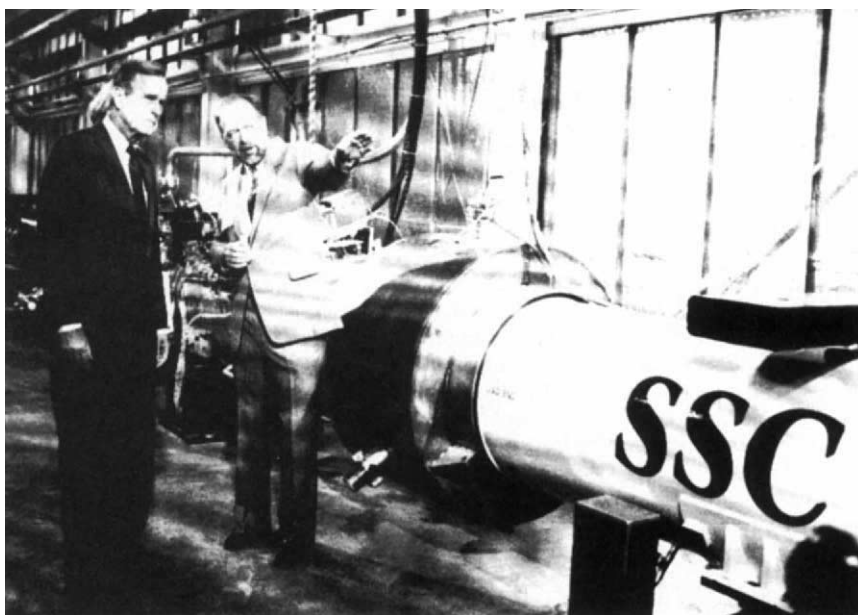
Two days before the vote in the House, an article critical of the space station appeared on the opinion pages of *The Washington Post*. Coauthored by biologist Maxine Singer, the president of the Carnegie Institution and a former NIH researcher, and Donald Brown, president of the American Society for Cell Biology, the article predicted that "the biology done on the space station will [likely] turn out to be a whopping and very expensive bore". The next day, Goldin replied in print that "space-based research in the life sciences and biotechnology will revolutionize our way of life in the 21st century", and he rhapsodized about "the possibilities for life science research with a full-time, state-of-the-art laboratory aboard Space Station Freedom".

On the same day that Goldin's article appeared, Healy wrote to him that she was "particularly dis-

turbed by the implication that NIH views future space experiments as critical to the overall success of biomedical research". She reminded him that she had told Congress last autumn that "it is unclear precisely how the unique space environment might be used to advance research on conventional health problems". And she warned Goldin that his comments, if not revised, could do "a serious disservice ... by the creation of unrealistic expectations and overpromise".

Healy's comments are unlikely to alter the NASA administrator's rhetoric, nor derail the space station; their real audience is the biomedical community, and the letter shows them that Healy is on their side. But the verbal battles, waged in public, also demonstrate the difficulty of maintaining a united front when times are tough. When budgets are at stake, the first rule is every discipline for itself.

Jeffrey Mervis



Director Roy Schwitters shows President Bush the magnets for the Superconducting Super Collider (SSC) during his visit last week to the Texas laboratory. Earlier this week, the Senate agreed to spend \$550 million in 1993 after voting down a proposal to kill the project.

As disappointing as the NSF votes were, they were overshadowed by the attacks from the biomedical community on NASA for its repeated claims of potential breakthroughs from research in space in the fight against a host of diseases, including cancer and AIDS. For more than a year, the American Physical Society has led a campaign to defeat the \$40-billion space station, arguing that its scientific contribution will be minimal and that the money could be much better spent on Earth-bound research.

The argument has so far failed to sway Congress: on 29 July the House voted 237:181 to reject an amendment to kill the space station, a margin that differed little from the 240:173 vote last year on the same amendment. But NASA's decision to highlight the life sciences has enraged biomedical researchers already upset that NIH's budget, at \$9 billion, is not growing fast enough to keep up with the scientific

Academy's letter to Russian raises issue of political views

Washington. The US National Academy of Sciences has taken the unprecedented step of asking one of its members to resign because of his personal views, triggering an international debate about the role of politics in recognizing scientific achievement.

In a letter dated 16 July and made public last week, the academy's president, Frank Press, and its foreign secretary, James Wyngaarden, express the academy's "strong aversion" to the antisemitic sentiments in a 1982 book by the eminent Russian mathematician Igor Shafarevich. The letter also asks Shafarevich to respond to charges that he has blocked the appointment of qualified Jewish mathematicians at the Steklov Institute in Moscow, which he once directed and where he is now head of the algebra section.

"Using your position to speak against Jews because of your perception of their influence upon Russian society is not only deplorable, but violates the principles of our Academy," the letter says. "If *Russophobia* represents an accurate expression of your views, and if our information of the composition of the algebra section is a reflection of your influence on hiring and appointment practices, you may wish to consider whether it is appropriate for you to maintain your membership of the National Academy of Sciences."

This is the first time in the 129-year history of the academy that a member has been asked to resign. Press says that there is no mechanism for dismissing a member.

However, not all who object to Shafarevich's words and deeds think that the academy is doing the right thing in asking him to leave. Two French members have suggested that Press resign for overstepping his authority, and others believe that the academy's letter was ill-advised.

"It was my understanding that election to the Academy was on the basis of scientific contribution, and I object to the injection of political criteria in asking someone to resign," says Serge Lang, a mathematician at Yale University. "While someone may deserve the opprobrium of the scientific community — which Shafarevich does for *Russophobia* — that does not affect his scientific contribution."

William Jaco, executive director of the American Mathematical Society, says that while he deplores Shafarevich's politics, he has "tremendous respect for his mathematics".

Shafarevich, a foreign associate member of the academy since 1974, was unavailable for comment and has not formally responded

to the academy's letter. Ironically, he was promoted for membership after writing *Socialism as a Phenomenon in World History*, an anti-government book that was seen as a brave act in the political climate of the mid-1970s.

The book attacks socialism on religious rather than economic or political grounds, advocating a return to a Christian and culturally orthodox Russian tradition that predates the Soviet revolution. The publication of *Russophobia* in 1982 continued this theme, but also introduces Shafarevich's concerns about the influence of Jews on Russian society.

One of the prime movers in the academy's decision to act is Lawrence Shepp, of AT&T Bell Laboratory, who has translated the book into English. Shepp says that *Russophobia* has fed existing antisemitic tendencies among the anti-democratic movement in Russia, as well as demoralizing Russian Jews.

Joseph Birman, a theoretical physicist at City University of New York and a frequent visitor to Russia, says that Shafarevich "is very visible in the public domain as a supporter of extreme nationalistic, racist and right-wing groups," and that Shafarevich's status as a renowned mathematician gives credibility to his views and to those with whom he associates. His election this spring to full membership of the Russian Academy of Sciences (see *Nature* 357, 617; 1992) is seen by some as an official endorsement of his opinions.

Henri Cartan and Jean Pierre Serre, two distinguished French mathematicians who are foreign associate members of the US academy, have contacted Press to deplore his letter to Shafarevich. Serre's letter refers to the issue as a "politically correct witch hunt" and suggests that Shafarevich be sent an apology and that Press himself should consider resigning.

In a reply to Cartan and Serre, Press says that the academy applies no test of political correctness to membership. "Since freedom of expression is a basic tenet of both science and democracy", he says, "it is appropriate to ask Shafarevich if his views and practices are consistent with non-discrimination against a particular ethnic group."

For Shepp, the real fight against Shafarevich will be waged in Russia. He wants the academy's letter, and other open letters that have been written to Shafarevich and published in the United States, to be published in Moscow as part of an effort to inform all segments of the former Soviet Union.

Ian Mundell

New German budget aims to restore eastern science

Munich. In the face of concern about the high cost of reunification, cash-strapped Germany is sticking to its decision to equalize research levels in its eastern and western states as soon as possible. The 1993 science and technology budget announced last week provides a generous sum for strengthening the research base in the former East Germany. But although research has done better than most in the national budget, the overall increase still lags behind inflation, and cutbacks in many areas seem inevitable.

Heinz Riesenhuber, the research minister, has persuaded the German federal government not to abandon research as part of its austerity programme by arguing that increased spending on research will stimulate growth in all areas. Research and technology programmes, mostly outside universities, have received an increase over 1992 of 3.8 per cent, nearly twice the average. Around 40 per cent of the country's overall research budget of DM9.6 billion (US\$6.3 billion), a slightly higher ratio than last year, is devoted to high-level basic research.

The eastern states of Germany will be the major beneficiaries of shifts in next year's budget: DM1.75 billion is earmarked for improving their research base, a 9.4 per cent increase over last year. Most of this money will go to large research establishments, but some will be used to improve the infrastructure of research in universities and to subsidize small companies.

The budget also provides the first signs of a shift in Germany's pattern of research support. By providing 8 per cent more money for ecology and climate research and by sharply reducing spending on nuclear and fossil-fuel energy research, the budget conforms to the results of a two-year investigation and to promises made at the Earth Summit in Brazil. In addition, large research institutes will be orientated more towards biological, rather than physical, disciplines.

Space research has also been given an above-average increase. Although national space programmes are to be squeezed, the budget increases the size of Germany's contribution to the European Space Agency (ESA) by 9 per cent, to DM1.2 billion. But the government hopes to save on this sum in negotiations with ESA's other member countries. The budget also gives priority to new, intelligence-based technologies and collaborative projects with former Eastern Bloc countries, hoping in particular to gain from Russia's expertise in space.

These priority projects will take money from a pot already diminished by inflation. As a result, the losers face the possibility of layoffs or the closing of facilities.

Alison Abbott

Cost of cleanliness may sink European Mars mission

London. The European Space Agency (ESA) says that it may not be able to afford a mission to Mars unless an international committee lowers standards intended to protect other planets from contamination from Earth. The ESA budget is so tight that the cost of sterilization regimens and other measures needed to prevent Earth organisms from reaching the planet could reduce the scientific payload or the number of probes; either result would make it harder for the Mars mission to compete against other possible ESA missions.

International guidelines have changed several times since the Viking probes landed on Mars in 1976. COSPAR (the Committee on Space Research), organized by the International Council of Scientific Unions, which has responsibility for setting standards of planetary protection, is expected to relax the rules further when it meets later this month at the World Space Congress in Washington, DC. But the change may not be enough for ESA's Marsnet.

Marsnet, one of four proposals being considered for ESA's next medium-sized mission, would land three physically unconnected but functionally complementary probes on the martian surface to study seismic and atmospheric conditions. The design incorporates as few protection measures as possible; essentially, it provides that the probes will be as clean as possible, but that only surfaces that will come into direct contact with Mars need to be sterilized.

Spending money on further protective measures will reduce the amount of science that can be done and will rule out a possible fourth probe. Although scientists have not determined an exact price, a full bioshield such as that used by Viking would breach

the \$300-million ceiling set for medium-sized missions.

Marsnet is the only candidate mission involving a planetary landing. Of the others, PRISMA is a helio-seismology project, INTEGRAL is a gamma-ray astronomy project and STEP is a fundamental physics project that will test the principle of equivalence. A decision will be made next June, with a tentative launch in 2001.

Complementary to Marsnet is a proposed mission by the US National Aeronautics and Space Administration (NASA), called MESUR, to land between eight and twenty-three probes on the martian surface. Although it is subject to the same COSPAR regulations as ESA, MESUR features protection similar to that used on Viking.

The Viking mission searched for life on Mars: apart from anxiety about introducing Earth organisms to Mars, researchers had to be sure that any life forms detected by the Viking probes did not come from Earth. Each probe was sealed in a box before the launch and baked at 112°C for 40 hours. According to NASA's John Rummel, the cost of the planetary protection is not a factor in the MESUR project.

The first of the Mars landing missions, assuming the money is available, will be the Russian Mars 94 project. It is not known just what planetary protection measures the Russians are planning, but they have promised to abide by COSPAR rules. The mission consists of a large orbiter with a very heavy scientific payload and two landers, which may penetrate the surface.

The consensus view is that Mars, believed to be the only extraterrestrial planet capable of supporting life (indigenous or otherwise), needs special protection. Even

if the scientific aims are not primarily exobiological, there are good arguments for keeping up standards of planetary protection. If a probe discovered a site with a temperature profile, water content or level of oxidation that would be friendly to life, its findings could be thrown into doubt by the question of contamination. On a larger scale, organisms deposited on the surface may be spread over a large area by the martian dust storms.

Although these possibilities are considered remote, delegates to the COSPAR meeting are expected to exercise caution. A panel of the US National Academy of Sciences is completing a study commissioned by NASA that Rummel expects will recommend looser requirements for probes landing on the martian surface but continued caution about those going beneath the surface.

Less is known about conditions beneath the martian surface, but the thinking is that they might be similar to those around Earth volcanoes. Any evidence collected by the proposed missions that demonstrates life-supporting conditions on Mars will once again raise standards for planetary protection.

Ian Mundell

ICI splits in two, forms drugs company

London. The British conglomerate Imperial Chemical Industries (ICI) unveiled plans last week to put most of its research-intensive divisions into a separate company. Pharmaceuticals, agrochemicals, seeds and specialty chemicals would join to form ICI Bioscience, while the company's other interests — paints, materials, industrial chemicals, explosives and surfactants — will remain as ICI. This demerger, as it is being called by ICI, is expected to be confirmed at a meeting of shareholders early in 1993.

In 1991, ICI as a whole spent £692 million (US\$1.3 billion) on research, of which 70 per cent was carried out by the divisions that will go to form ICI Bioscience. With current revenues of £3.9 billion, ICI Bioscience would become the world's ninth largest drugs group, although commentators have noted that much of its sales comes from agrochemicals. The remaining bulk-chemicals company would drop from fifth to seventh in the world.

The financial and the scientific communities welcomed the news: the consensus is that a company focused on research will be better able to refill a pharmaceutical pipeline drying up over the next three to four years. Frank Kaye, a senior partner in PA Consulting, an international science consultancy, points out that the demerger combines all the genuinely global parts of ICI. "Pharmaceuticals and agrochemicals can get on with being international businesses now that they are away from the anglocentricity of the bulk chemicals divisions", he says. **Ian Mundell**

Alison Abbott

EC asked to supplement Framework

Munich. Filippo Pandolfi, head of the European Commission (EC) research programmes, has asked for an extra ECU1.6 billion (US\$2.15 billion) to continue research and development projects now that a delay in the next five-year programme, due to start in 1993, seems inevitable. The funds would be used to support the EC's current Framework research plan, which ends in 1994, and to ensure that funding for applied research does not dry up. No change in funding of biomedical research is expected, and there will be no expansion of the human capital and mobility schemes.

The next programme is expected to be a victim of increasing bureaucracy within the commission. A legal challenge earlier this

year to the commission's decision-making process has led to new procedures intended to ensure that any decision is signed by each of the 17 commissioners. Ratification of the Maastricht Treaty will give the European Parliament a greater say in community policy, so that it will take even longer to reach consensus. And a broadening of the EC's research responsibility beyond its present aim of strengthening the science and technology base of industry to include environmental programmes will also require a revision of the programme.

The EC Council is expected to adopt Pandolfi's proposal in a decision to be made before December.

Alison Abbott

Biosphere 2 project told to make room for science

Washington. Biosphere 2, the \$150-million artificial ecosystem operating in the Arizona desert, may indeed be a grand experiment in living harmoniously with nature. But a new report from an outside panel says that the project will not generate any useful scientific results until it hires a scientific director, designs and carries out a research plan and makes its data available to outside scrutiny.

IMAGE
UNAVAILABLE
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REASONS

Biosphere 2 — can it be more than a tourist attraction?

An eight-member committee of environmental scientists, chaired by ecologist Thomas Lovejoy of the Smithsonian Institution, was asked to describe what Biosphere 2 must do to make a meaningful contribution to science. The report does not say what type of research should be conducted nor how much it would cost, but Lovejoy praises the project as "an act of vision and courage" that will "make important scientific contributions in the fields of biogeochemical cycling, the ecology of closed systems and restoration ecology" if the panel's recommendations are carried out. The project has already produced results that would otherwise have been impossible to obtain, he says.

The story of Biosphere 2 is one of great hype followed by a certain amount of disillusionment. The idea was to set up a self-sustaining, artificial environment in which a number of people — or bionauts — would be sealed for two years. The aims were to generate scientific knowledge, to educate the public and to make a profit through tourism and new technology.

However, those involved in the early stages of the project say that science was low on the agenda in terms of both money and the interests of its organizers. The credibility of the project was further damaged when it was revealed that Biosphere 2, which began to operate last September, was not as

self-sustaining as it was made out to be — supplies and air were being brought into the supposedly sealed environment and a carbon dioxide scrubber had been installed.

The success of the committee's suggestions, and the amount of money to be invested in basic research, depends largely on who is appointed as scientific director. Lovejoy estimates that the report's recommendations could be implemented for a few hundred thousand dollars. The company behind Biosphere 2, Space Biosphere Ventures, says that it was considering the appointment of a scientific director before the committee submitted its report.

The committee recommends that the director be a respected researcher with responsibility for coordinating the scientific activities of Biosphere 2, augmenting and facilitating new projects, developing the scientific staff and engaging with the scientific world at large. Given the view of some people that those concerned with the project are either benighted idealists or cultists aiming at world conquest, a considerable research budget and visible independence will certainly be needed to attract a reputable scientist.

The report is critical of the project's current approach to research. It says that Biosphere 2 must stop treating all scientific information as proprietary and begin to publish its results in peer-reviewed journals. It also needs to revise its data collection and storage system and take steps to ensure that all the data collected are properly validated.

One problem not so easily fixed is the scientific capabilities of the eight bionauts. With one exception — a gerontologist — none has PhD-level scientific qualifications. The report says that outside researchers should be enlisted to make up for this shortcoming. There also needs to be a movement of materials such as tissue or water samples in and out of the sealed environment.

Edward Bass, a Texas businessman whose philanthropy has made the project possible, says only that the concerns and recommendations of the committee will be "immediately and fully addressed".

Ian Mundell

Soviets reported to have dumped nuclear waste in Arctic

Washington. Scientists from the United States and other countries are looking into reports that the Soviet Union dumped vast amounts of radioactive waste into the relatively shallow waters bordering the Arctic Ocean. The material is said to range from the intact reactor of a nuclear submarine to barrels of radioactive waste from nuclear-powered icebreakers.

Members of the Russian Parliament have asked their government to release data about the dumping, which is alleged to have occurred in the shallow Karas and Barents seas off the northwest coast of Russia between the 1960s and last year. Last month, researchers and policy-makers from Canada, Russia and the United States met at the Woods Hole (Massachusetts) Oceanographic Institution (WHOI) to devise strategies for obtaining more data, including a conference next June in Canada to which Russian experts would be invited.

The researchers need to know the location, amount and packaging of the waste, as well as precisely what the Soviets dumped, before they can assess its possible effects. Officials from the former Soviet Union have released little information about the dumping, hampered by the decentralized and classified nature of the data.

"I can imagine that it's very difficult to find the information, because sometimes decisions were made by a few people", says Raphael Vartanov, a senior research fellow at WHOI's Marine Policy Center on leave from the Russian Academy of Sciences. Vartanov and others suspect that some bureaucrats are withholding data for fear of taking the blame or because they "still think in the old way".

Not content to wait for information, scientists have also organized expeditions to monitor known dump sites. A Norwegian-Russian research team that also includes one US scientist is due to leave next week, and several US agencies are planning an international expedition in the autumn to take measurements at sea and on land.

Although few signs of extensive nuclear pollution have appeared in water leaving the Arctic, scientists are concerned that the effect of the alleged dumping could be magnified by the fact that it took place in seas only 60 to 300 metres deep. Such shallow waters harbour most of the ocean's organisms and are more turbulent than deeper ocean basins. Researchers also want to examine the water circulation around dump sites.

"I certainly don't think there is an ecological catastrophe happening", says Hugh Livingston, a senior research scientist at WHOI, "but I'm concerned for the future."

Traci Watson

Bifurcated Belgium shows the strain from collaboration between cultures

Brussels. As the European Communities (EC) move towards a unified Europe, it is ironic that Belgium, in whose capital their headquarters is housed, is consolidating its own division into separate regions. But the path to federalization has not been smooth, and Belgian science now suffers the consequences of a loss of cooperation across the cultural divide.

Like many of the countries in Europe now splitting into ethnically defined fragments, Belgium is a product of recent history. Flemish-speaking Flanders in the north and French-speaking Wallonia in the south were thrown together in the 1830s to be a buffer between France and Germany. But the artificial boundaries caused economic and cultural tensions, and in 1988 a federal system was created.

Three semi-autonomous regions — Wallonia, Flanders and bilingual Brussels — were founded, with Flemish speakers outnumbering French speakers by 55 per cent to 38 per cent. Responsibility for a large part of the nation's science policy has shifted quickly from the national government to the regions and cultural communities.

Belgian research is planned within three broad areas. The national government directly funds some programmes, such as the BFr500 million (US\$17 million) AIDS programme, that are in the national interest. This year, about BFr12 billion has been set aside for this purpose. The regions control economic policies and handle applied science and technology while the communities, which are responsible for all cultural issues, deal with basic research.

Flanders and Wallonia operate as two countries. Budgets are divided roughly 55:45 according to a complex formula involving population, fiscal revenue and extent of territory. This also applies to the National Fund for Science Research (NFSR), whose two administrations are strictly separate. A French speaker cannot apply for a grant from the Flemish NFSR, nor can a Flemish scientist ask for French funding.

But the two parts do share the 40-odd,

discipline-orientated 'science committees' that assess grant proposals. These committees are usually composed of five Flemish-speaking and five French-speaking scientists plus two foreigners who must be able to speak at least one of the

federalization has undermined established systems for choosing the best science. Review panels tend to fragment into their ethnic groups, and members show little interest in commenting on grant proposals from the opposite community. The result is half the desired input into the process.

At the most recent meeting of the physiology and pathophysiology committee, for example, the French reviewers were totally absent from discussions about Flemish proposals, and vice versa.

Federalization has also changed the way in which national funds are distributed. Scientists complain that decisions are now made by politicians. The new Flemish government has promised to reduce the influence of the national government, but so far this has not happened.

Scientists are also unhappy that a larger share of the research budget is going directly to universities in the two communities on the basis of their enrolments rather than the quality of their research. Since 1987, the amount of research money given to universities in Flanders for their own allocation has risen by more than a third to

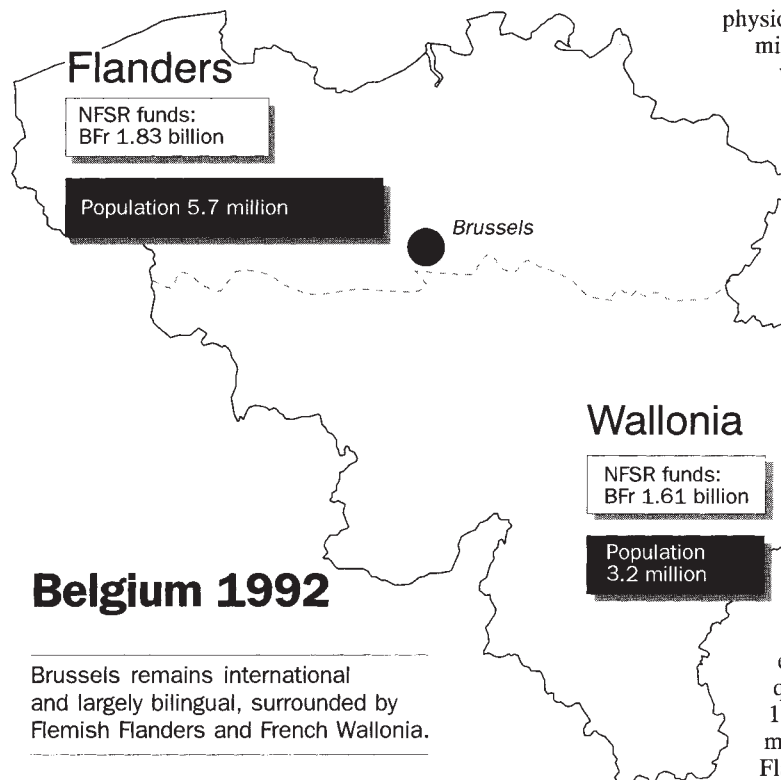
BFr379 million.

Walter Fiers, a molecular biologist at the University of Ghent, says that most universities, to keep their departments happy, tend to allocate the money evenly, and do not use a standard scientific peer-review system. "Science is so important and so expensive that its funding should be selective, not democratic", he says.

But Claude Truffin of Belgium's National Ministry of Science says that many of the problems are just a consequence of settling in. Although admitting that the regions and communities are not cooperating well and that the national government is failing to coordinate activities, Truffin says that "we may not have set up a very efficient and well-harmonized system, but there is nothing stopping us from moving in that direction".

Despite complaints about the system, there are few calls for radical change. As a consequence, it seems likely that the process of "settling in" may take a few more years.

Alison Abbott



languages. According to Jos Traest, general secretary of the Flemish side, this mix should allow decisions to be made independently of financial interests.

Why then is the system not working? There are two main problems. First, the lines of authority between the three levels of government are unclear, making it hard to reach decisions. In addition, large-scale collaborations between French and Flemish speakers have become difficult to arrange. Marcus Bogaert of the J.F. & C. Heymans Institute in Ghent once had a regular collaboration on ageing and disease states with a French-speaking group at the Catholic University of Louvain, but decided this year that the process had become 'so cumbersome that it was not worth the bother'. French-speaker Arsène Burny, a molecular biologist at the University of Brussels, says that it is easier to 'forget your neighbour 30 km up the road' and arrange an international collaboration; the process is no more complicated and the result carries more prestige.

Second, scientists feel that the process of

Physical sciences dominate Japan's special grant awards

Tokyo. The physical sciences and technology have moved ahead of biology on this year's list of grants to individual scientists announced last week by the Japanese Ministry of Education, Science and Culture. Researchers in physics, chemistry and astronomy received seven of the 11 awards in the ministry's 'special distinguished' grant category, a competition that has been dominated by the life sciences during most of the past decade.

The grants, worth about ¥100–¥300 million (US\$750,000–\$2.2 million) over three to five years, are meant to support "internationally renowned research that is likely to give outstanding results". They provide an indication of where some of Japan's best basic research is being done.

One of the largest grants — ¥283 million for five years — goes to Toshimitsu Yamazaki of the Institute for Nuclear Study of Tokyo University. Yamazaki will use the low-energy antiproton beam at the European high-energy physics laboratory (CERN) in Geneva to study anomalously long-lived hadronic atoms, such as antiprotons embedded in helium. The grant will further strengthen the collaboration between CERN and Japan's leading national university.

Daiichiro Sugimoto of Tokyo University's department of Earth science and astronomy, who has developed a high-speed

computer to model evolution of star clusters and galaxies (*Nature* 345, 33; 1990), gets ¥189 million for five years to upgrade his computer to carry out teraflop calculations of such many-body problems. Sugimoto intends to model not only the behaviour of stars and galaxies but also the molecular dynamics of, for example, proteins.

Another user of super-high-speed technology is Akira Hasegawa of Osaka University's department of communications engineering. He receives ¥185 million over five years to test an optical fibre transmission system based on the idea that solitons can transmit vast amounts of multichannel data (40 gigabits) over tens of thousands of kilometres.

In contrast with previous years, only four awards went to those in the biological sciences. But two of them are among the largest. Tasuku Honjo of Kyoto University receives ¥184 million over three years to elucidate the molecular and regulatory mechanism of programmed cell death, an important element in understanding embryogenesis and autoimmune diseases. And Nobutaka Hirokawa of Tokyo University faculty of medicine receives the largest grant — ¥290 million for four years — to study the molecular architecture, dynamics and function of the neuronal cytoskeleton.

David Swinbanks

Bush delays new risk guidelines

Washington. A US proposal to relax standards to assess the risk posed by chemicals in food and in the environment has been delayed by the Bush administration, disappointing those who believe that current methods are outdated and imprecise. Last week's decision could have the unintended effect of killing the plan: Bush's challenger in the forthcoming presidential election, Democrat Governor Bill Clinton, is said to be opposed to the notion of easing restrictions.

The decision is the latest move in an extended government debate over how to assess risk. Many researchers believe that current models err so far on the side of safety that chemicals posing nearly negligible risk are either banned or subjected to costly regulation.

Traditionally, each federal agency chooses its own risk assessment methods. But the proposed executive order would have created an independent panel of scientists working out of either the White House or the US National Institutes of Health to set

risk assessment procedures for all federal agencies. The order would also have permitted new assessment methods allowing chemicals that pose little risk at low levels.

According to the White House, the executive order has been held in abeyance because Bush administration officials could not pay it proper attention during a presidential campaign. Others point to more partisan politics: environmental and consumer groups represent a constituency that cannot be ignored in a close election.

If President Bush wins in November, he is expected to revive the proposal. But if Clinton wins, many say he would not undercut his support among environmentalists by revising risk assessment.

"I think they missed the opportunity to bring the weight of scientific opinion to bear" on risk assessment, says Aaron Wildavsky, a political science and public policy professor at the University of California, Berkeley, who believes that Clinton will win the election. "They've let it drop, and now it's gone."

Traci Watson

NEWS IN BRIEF

Washington. The European space probe Giotto has been put to electronic sleep and is heading back to Earth after its successful encounter last month with comet Grigg-Skjellerup. But the seven-year-old spacecraft, which flew by comet Halley in 1986, has probably carried out the last of its scientific duties.

Although Giotto is projected to come within 220,000 km of Earth in 1999, it is unlikely to pass near another comet. Even if it did, it probably lacks the fuel to manoeuvre into position to make observations. Finally, European Space Agency officials are not sure that they can afford to maintain for the rest of the decade the groundbased hardware used to communicate with Giotto. **J.M.**

New Delhi. The Indian government says that the Russian space agency has promised to honour a contract to purchase cryogenic rocket engines that has triggered a boycott by the United States.

"The contract between ISRO [Indian Space Research Organization] and Glavkosmos remains in force and is being implemented," India's science minister, P.R. Kumaramangalam, told parliament last week. He said that Russian officials have "reassured" India that they will carry out the contract. India has already paid Russia more than a third of the \$80 million it will cost to acquire two engines and the accompanying technology.

The United States believes that the contract violates an international agreement to prevent the spread of nuclear weapons technology, but India and Russia insist that the technology will be used only for civilian purposes. The rockets will be part of a geostationary launch vehicle to be tested in 1995. **K.S.J.**

Washington. Denmark's Medical Research Council has released the country's first plan for dealing with allegations of scientific misconduct and ethical violation in research. Detailing eight well-known cases of misconduct in the United States and other countries (but none in Denmark), the council said that a contributing factor was the lack of established procedures for dealing with such abuses in those countries. It recommended new guidelines on clinical research ethics and conflict of interest, and the creation of a national committee to review charges of misconduct.

The committee, which will be functioning by October, will have a high court judge as chairman and six health science researchers as members. When appropriate, the committee is expected to set up special *ad hoc* committees to investigate specific cases. The report also recommends that scientists under investigation be given the right to representation by an attorney, appeal, cross-examination and examination of the data. **C.A.**

Sex testing at the Olympics

SIR — During the 1992 Olympic Games at Albertville, a number of geneticists expressed serious doubts about the expediency of sex chromosome testing.

Barr's method has been used by the International Olympic Committee (IOC) and the International Amateur Athletic Federation (IAAF) since 1967. Because of its known shortcomings, in particular regarding XXY type abnormalities, the IOC replaced it with a method based on amplification of the Sry gene¹, used for the first time during the Albertville Games in France². The absence of the Sry gene seems to be a key element in determining the female sex, although it obviously cannot replace all the genetic, hormonal and phenotypic criteria that characterize gender³.

As the IOC's sole aim is to find an efficient solution to the practical problems of fraud screening before competitions, tests should be assessed with this in mind.

Only athletes competing in women's events are screened for fraud, as a woman winning in a male event is unheard of. The amplification of the Sry gene may fail to recognize certain XX men, but what are the chances of coming across such a rarity in an Olympic competition? The problem of women who are insensitive to androgens (1 in 20,000, XY subjects currently classified as male hermaphrodites) clearly illustrates the limits we set to our mission. Contrary to some reports, this test has never been used by the IOC to disqualify athletes. It simply leads to an in-depth study by a medical commission which makes its ruling on the merits of the particular case.

The unique practical tests we carried out on 557 athletes at the Albertville Olympics led us to the following conclusions.

The issue of gender verification itself is not at stake. It excludes athletes who do not satisfy the requisite criteria to compete in a women's event and also protects athletes whose physical appearance might give rise to suspicion of fraud.

Thanks to the use of buccal smears and the stability of DNA, the test is practical to perform. Much greater reliability (over 99 per cent) is achieved than with possible alternatives, past or present. In the context of intense physical effort, hormonology would not be reliable and would solve neither the problem of androgen insensitivity nor other controversial cases. As for the direct examination of the genitals proposed by the IAAF⁴, there is a risk of certain cases of transsexualism mentioned by Ferguson-Smith⁵ being missed. Furthermore, most athletes agree on one

point: a gynaecological examination for purely sporting purposes is a traumatic experience.

The IOC has therefore decided to continue using sex chromosome testing while improving its reliability. Because this test is likely to deter anybody tempted to cheat, the test should lead to an in-depth medical examination only in rare cases. This strict method, used for the first time at Albertville, is welcomed by athletes and is likely to receive wider acceptance in the future.

B. Digeon

Département de Biologie

P. Hamon

M. Robert

Département d'Endocrinologie,

Centre Hospitalier BP 1125,

73011 Chambéry Cedex, France

P. Schamasch

COJO,

73206 Albertville, France

M. Pugeat

Département d'Endocrinologie,

Centre Hospitalier,

Universitaire Antiquaille,

69005 Lyon, France

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4. Ljungqvist, A. & Simpson, J. L. *J. Am. med. Ass.* **267**, 850–852 (1992).

5. Ferguson-Smith, M. A. & Ferris, E. A. *Br. J. Sp. Med.* **25**, 1, 17–20 (1991).

AIDS causation

SIR — John Maddox (*Nature* **358**, 13; 1992) takes *The Sunday Times* to task for reporting that anal intercourse was present as a risk factor in the case of the Birmingham haemophilic who appears to have infected four women with human immunodeficiency virus (HIV). Why is a journal dedicated to science so afraid of facts?

The health authority in Birmingham started a national panic on the basis that transmission of the virus had resulted from "straightforward heterosexual intercourse". It declared that other risk factors had been ruled out. Our inquiries produced three relevant findings:

(1) There had been minimal investigation into the circumstances in which HIV had been transmitted, so the assurances given to the press that no other risk factors were involved were unfounded.

(2) The young woman who died of pneumonia was admitted to hospital in such a breathless state that she had to be sedated and put on a ventilator immediately. There was no chance to question her about her lifestyle before she

died. Neither her parents nor her long-term boyfriend had been approached by health officials to learn more of the circumstances of her death. All she had told the doctors was that she had been out with a haemophilic, on which basis she was tested for HIV antibodies and found to be positive — hence the AIDS diagnosis.

(3) The haemophilic at the centre of the affair had anal intercourse "more often than not", according to one of his former girlfriends, and two others said they had had intercourse with him this way.

At no point did we suggest that the hazards of anal intercourse were novel. The "new evidence" referred to in our headline concerned the above. Nor did we deny the possibility of HIV transmission through normal heterosexual intercourse. We quoted Professor Gordon Stewart as saying that the transmission rate with that method of intercourse was low.

You accuse us of putting consistency before correctness, but where is the incorrectness in what we reported?

You are also absolutely wrong in calling *The Sunday Times* a convert to Peter Duesberg's view that HIV is irrelevant to AIDS "and the comforting corollary that people have nothing to fear from heterosexual intercourse".

We examined Duesberg's ideas and decided that his challenge to the conventional view of HIV as the cause of AIDS was worth reporting, for the first time, in a national newspaper, along with that of the 50-strong "Group for the Scientific Reappraisal of the HIV/AIDS hypothesis". We stand by that decision. It does not mean we accept his alternative views on AIDS causation.

In the same issue in which we reported Duesberg's challenge, we carried a two-column interview with Professor Luc Montagnier, in which he made clear his own view that "without HIV, I don't think we would have AIDS epidemics", though he also declared his belief that there are some AIDS cases in which HIV plays no part. The following weekend, we reported on the work by Professor Angus Dalgleish and others to find a treatment based on the theory that HIV triggers an autoimmune reaction leading to AIDS.

We do however consider that Duesberg has raised some important questions. His claim that mainstream science has climbed on an HIV bandwagon which it defends in an unreasoning way is supported by your unwarranted attack on our coverage.

Neville Hodgkinson

(Science Correspondent)

The Sunday Times,
1 Pennington Street,
London E1 9XW, UK

Beyond the nation-state

SIR — Andrejs Baidins¹ has a naive faith in the institution of the nation-state if he believes that a multiplicity of them will result in “an unprecedented leap of progress”. In a curious lapse into social Darwinism he asserts that “[This] is the way of nature . . . more diversity, more cultures, more languages, more nations”, the “competition” between which will be a driving force for progress. I do not here wish to address the fallacies of social Darwinism, but I wish to make three other observations on Baidins’s pro-nationalist thesis.

(1) If biological analogy is relevant, the history of life on Earth is more a history of evolving ‘federalism’ than the reverse. Thus, some 1.5 billion years ago, simple prokaryotic cells came together to form ‘federal’ eukaryotic ones, and almost a billion years later these eukaryotic cells began to form the vastly larger ‘federal’ entities we know as multicellular animals. Many of these have in turn evolved ever more complicated social structures, which have resulted in even larger ‘federal’ communities based on the co-operation of many individuals.

(2) In particular, the species *Homo sapiens* has, over the past 50,000 years (and more or less in the following order), evolved political institutions appropriate for hunting and gathering, village agriculture, city states, military empires, geographically limited nation-states, and continental-sized federal states (a process begun at Philadelphia in 1787, and continuing to Maastricht in 1991). Each step in this political evolution, while resulting in fewer independent political units, has nevertheless increased the potential for human progress. Thus, in the third millennium BC, the Sumerian cities were able to undertake projects (for example, temple construction and canal building) utterly beyond the abilities of their still-neolithic neighbours, while, in our own day, a federal continent such as the United States is able to take on projects beyond the abilities of old-style nation-states such as Britain or France (the landing of man on the Moon is an obvious example).

(3) While the disadvantages of “competition” between nation-states are obvious, owing to the inherent risk of military conflict, it is actually very hard to identify the benefits hinted at by Baidins in his letter. While there are a handful of technological inventions (for example, radar and jet-propelled aircraft) that have appeared earlier as a result of military conflict than they might otherwise have done, this is not generally true of technological progress. What was the role played by international

competition in the invention of the steam engine, for example, or of the dynamo, or of radio transmission, or of the airplane? None of these key inventions was developed by nations engaged in competition with other nations, but by individuals who were not obviously motivated by nationalistic considerations.

In short, there is little evidence that nations and nationalism have had a positive influence on human progress, and, if we consider all the pointless wars that have been fought between them, their net affect would seem to have been almost entirely pernicious. These considerations led Kant² to conclude that nation-states “hamper progress towards [the] full development of man’s natural capacities”. Far from being seen as “the way of nature” within some social Darwinian world-view, nation-states should more properly be viewed as an intermediate step in the political evolution of human societies towards a world organized on federal principles. There are good reasons for believing that a federal world (complete with a federal government) would provide more opportunities for human progress than an anarchic world of competing nation-states.

I. A. Crawford

19b Clarendon Drive,
London SW15 1AW, UK

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Climate change

SIR — Wilfred Beckerman’s review of books on environment and economy (*Nature* **357**, 371; 1992) illustrates the continuing difficulties in the very theme he is writing about: the dialogue between scientific and economic disciplines. He raises many good critical points, but also reflects the tendency of many economic analyses to hide critical assumptions beneath a veneer of objectivity. He focuses upon work by Nordhaus as “probably the best attempt so far to estimate the whole schedule of costs and benefits” concerning climate change.

Nordhaus is a brilliant mathematical economist, but this does not prevent his estimate of the costs of climate change from being myopic speculation. The quoted analysis estimates the costs to the US economy of being in a warmer climate (one corresponding to “best estimates” of the impact of the doubling of atmospheric carbon dioxide), and extrapolates this globally. It does not consider the probable dynamics of the pro-

cess, for example the changing severity and frequency of unpredictable extremes such as prolonged drought. It assumes global fungibility (interchangeability) of different resources (yet people already die of water shortage, famine and flood despite immense wealth elsewhere), and it neglects the possibility of costly conflicts arising from changes in such resources. Using his point estimate of the damages from the doubling of atmospheric carbon dioxide, Nordhaus assumes a weak general relationship between temperature change and costs although his policy prescription could lead ultimately to atmospheric changes far beyond a doubling of carbon dioxide or anything observed in the geological record. Despite this and all the literature on the chaotic nature of climate and oceanic systems, his quantified estimate neglects any possibility of future surprises or other sudden changes in weather patterns.

Nordhaus’s key paper (*Econ. J.* **101**, 920–937; 1991) recognizes some of this and states that his quantification “is clearly incomplete . . . we might raise the number [cost of climatic change] to around 1% of total global income to allow for these unmeasured and unquantifiable factors, although such an adjustment is purely *ad hoc* . . . it is not possible to give precise error bounds . . . but my hunch is that the overall impact is unlikely to be larger than 2% of total output”. Beckerman should explain why an economist’s “hunch” about climate change should command more respect than the warnings of hundreds of scientists about the risks associated with human interference with the atmospheric heat balance, and of social scientists about the possible human impacts.

A crucial value of economics is that it can help us to understand trade-offs between different policy options. To do so, economists need to use assumptions regarding the possible costs and risks of climate change, and of technological options for abatement. When economists elevate their own hunches and simplifications above the judgements of scientists and technologists who specialize in them, it is a recipe for continuing the unfortunate divide between economics and other disciplines. Beckerman would have done better to cite another economist featured in the books he reviewed, namely David Pearce’s increasing emphasis on the fact that with large potential risks in a system — the planet — of such inertia and importance, good economic analysis prescribes caution.

Michael Grubb

Energy and Environmental Programme,
Royal Institute of International Affairs,
Chatham House,
10 St James’s Square,
London SW1Y 4LE, UK

Is charge quantization exact?

There is mild excitement at the prospect of using anti-hydrogen as a means of telling whether the charges of protons and antiprotons differ, and, if so, by how much.

If only the magnitude of the electric charges of the electron and the proton were slightly different, the expansion of the Universe would be accounted for at a stroke: electrostatic repulsion would blow it apart. That is what Einstein suggested in 1924. And the argument can be turned around to infer, from the known expansion of the Universe (the long-standing uncertainty in Hubble's constant notwithstanding) that the charge difference between electrons and protons cannot be large, for that would force the Universe to expand more rapidly than is observed. In other words, there is no obvious reason to doubt what the elementary textbooks say, that the charges of the electron and the proton are equal in magnitude but opposite in sign, so that an intact hydrogen atom is electrically neutral.

What this implies, in turn, is general support for the idea that electric charge is strictly quantized, in units of the elementary charge $\pm e$, where the electric charge of a proton is $+e$ and that of an electron $-e$. Although, when several nucleons are thrown together to make a nucleus, the total mass will be smaller than the sum of the masses of its parts (by the mass equivalent of the binding energy), the total charge is strictly the algebraic sum of the charges of the component nucleons.

The only known exceptions to the rule that electric charge is strictly quantized are the particles called quarks, but they are exceptions of the kind that may be held to prove the rule. Free quarks, with charges of $\pm\frac{1}{3}e$ and $\pm\frac{2}{3}e$, seem not to be capable of independent existence, but only in combinations (hadrons or mesons) whose total charge is either zero or an integral multiple of e .

So why should people such as R. J. Hughes (from the Los Alamos National Laboratory) and B. I. Deutsch (from the University of Aarhus) be concerned with the possibility that the quantization rule may not be strictly correct or, more accurately, with an attempt to estimate its precision? Their case (*Phys. Rev. Lett.* **69**, 578–581; 27 July 1992) is that although the equality of the charges of the proton and the electron has now been proved to the hilt, there is nothing like the same precision in the comparison of the electric charges of the corresponding antiparticles, the antiproton and the positron. They plead for measurements with anti-hydrogen to advance the cause.

The stimulation provided by Einstein's argument is easily appreciated. The ratio of the electrostatic and gravitational forces

between an electron and a proton, whatever their distance apart, is the huge number 10^{39} , which is simply another way of saying that gravitational forces are very much weaker than electrostatic forces. But that implies that the expansion of the Universe is enormously sensitive to any difference between the electric charges of the electron and the proton; Hughes and Deutsch quote R. A. Lyttleton and Hermann Bondi for the estimate that a charge difference of $10^{-20}e$ would account for the expansion of the Universe. But the precision of the measured difference is an order of magnitude better than that, or less than $(0.8 \pm 0.8) 10^{-21}e$. Given that the neutron is now known to be electrically neutral to within $10^{-20}e$, the electrostatic expansion of the Universe looks like a lost cause.

The interest of charge comparisons involving antiparticles, as Hughes and Deutsch explain it, is more subtle and even profound. What constitutional lawyers would no doubt call the enabling equations of antiparticles, Dirac's equation for example, predict that particles and antiparticles will have equal masses. If that is taken as a given, then it should be possible to make high-precision comparison of the charges of electrons and positrons, or of protons and antiprotons, simply by measuring their respective frequencies of revolution (the cyclotron frequency) in a suitable magnetic field, which is one of the everyday by-products of high-energy physics. But who says that the enabling equations are exact? Hughes and Deutsch argue that that amounts to the assumption of CPT invariance, or that the equations of motion for particles/antiparticles are invariant with respect to charge inversion, parity (or the inversion of space through an arbitrary origin of coordinates) and time reversal — a simple but not necessarily correct assumption about the Universe on a microscopic scale.

So how to compare the electric charges of particles and antiparticles without supposing that their masses are intrinsically equal? Strictly, measurements of the cyclotron frequency are measurements of the ratio of charge and mass, e/m . Plainly an independent measurement is needed if differences of e are to be separately determined. Hughes and Deutsch argue that the best source of further information must be a spectroscopic measurement of an exotic atomic state in which one of the constituents is an antiparticle.

The most obvious candidate is positronium, the atom whose constituents are an

electron and a positron. This has a ground state and excited states, just as hydrogen atoms do, but the energy levels are differently spaced because the particles have different masses. Technically, the Rydberg constant for positronium is almost exactly half that for hydrogen. So, given a cyclotron comparison and a measurement of a known energy level of positronium, it should be possible to put a limit on the difference of charge.

There are data in the literature sufficient to pin down the difference of the electric charges of the electron and the positron. The cyclotron frequencies of the two particles are known to differ by one part in 10^8 , while the respective Rydberg constants are in a ratio that differs from unity by less than 4×10^{-8} . There is a prospect that each of the component measurements may be made three orders of magnitude more precise, which would reveal differences of the order of 10^{-11} in the two charges. That will still be some way short of the precision with which the charges of the electron and the proton have been compared, but will no doubt not be sniffed at.

The comparison of the charges of the proton and antiproton is more difficult. The analogue of positronium for these particles is the bound state of a proton and antiproton, for which the available mass measurement is much less certain. Hughes and Deutsch argue that the precision of the comparison using data now available is merely two parts in 100,000, despite the much greater precision of the cyclotron measurements themselves.

So how to make progress? That is where anti-hydrogen comes in. The idea is that isolated atoms of anti-hydrogen would be relatively much more stable than those of positronium (whose constituents can mutually annihilate each other). Hughes and Deutsch calculate that it should be possible to measure the Rydberg constant of anti-hydrogen to one part in 10^{15} , and that a suitable cyclotron comparison of proton and antiproton and electron and positron would pin down the difference of charge of proton and antiproton to one part in 10^{11} .

But first catch some anti-hydrogen. Nobody has made a single atom yet, unless by accident. The ideal will be to prepare a handful of atoms in a suitably cooled ion trap, holding them there long enough for spectroscopy to be possible. It will be interesting to see which laboratory is the first in the field. While the search goes on, the doctrine of CPT invariance lacks underpinning, but at a level of precision unlikely to keep people awake at night.

John Maddox

Inducing concentric worm holes

Robert K. Herman

INDUCTION is the process in development in which the fate of one cell mass is determined by another. A simple example occurs during vulval development in the nematode *Caenorhabditis elegans*: a gonadal cell called the anchor cell induces three neighbouring cells to embark on a programme of cell division and morphogenesis, which culminates, in a few hours, in the formation of a vulva¹⁻⁵. On page 470 of this issue, Hill and Sternberg⁶ report strong evidence that they have identified the anchor-cell signalling molecule, which they find is a member of the EGF (epidermal growth factor) group of growth factors.

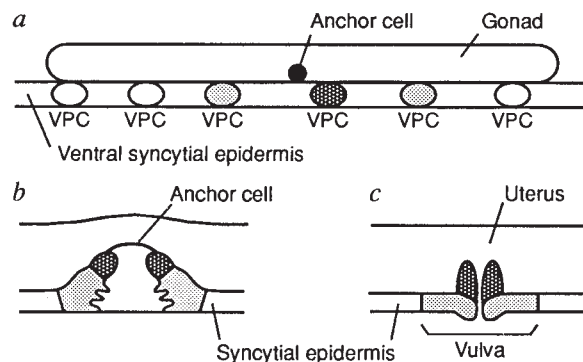
The vulva of *C. elegans* is formed during the late larval stages of hermaphrodite development. Each hermaphrodite produces oocytes which can be fertilized either by sperm manufactured internally or by sperm injected by a copulating male. The function of the vulva, then, is twofold: it provides an entrance for male sperm and an exit for fertilized eggs.

Each of six cells situated in a longitudinal row on the ventral side of the hermaphrodite is capable of generating descendants that contribute to the vulva. These six cells are called the vulval precursor cells (VPCs). The name, though, is slightly misleading because normally only three VPCs make a vulva, the remaining three taking an alternative developmental path: they divide once and then fuse with a large multinuclear epidermis that surrounds them and the developing vulva, and covers most of the worm's body except for the head and tail. The three VPCs selected for vulval development are those closest to the anchor cell; together they generate 22 descendants that undergo cell fusions and intricate morphogenetic movements to produce a vulva (see figure). The evidence that the anchor cell induces vulval development comes from experiments in which it has been removed, either by laser ablation or by mutation. All six VPCs then fuse with the surrounding syncytial epidermis and no vulva is formed.

Normally the same three VPCs are chosen for vulval development because they are invariably closest to the anchor cell. However, the ability of the other three to contribute to vulval development has been demonstrated by experiments in which a normal vulval-producing VPC has been destroyed by a

laser microbeam or the relative positions of the VPCs and the anchor cell have been changed by mutation; in these cases, a vulva may be formed from a different set of three VPCs. In addition to producing the inductive signal, the anchor cell becomes the focus of vulval morphogenesis and ensures that the vulval and uterine openings are concentric.

Hill and Sternberg⁶ propose that the anchor-cell signal is the product of the



The three vulval precursor cells nearest the gonadal anchor cell are induced to generate 22 descendants that undergo cell fusions and morphogenesis to produce a vulva. *a*, Third larval stage; *b*, fourth larval stage; *c*, adult. Patterns of shading in *b* and *c* correspond to regions contributed by ancestral VPCs shown in *a*. (Adapted from refs 3 and 4.)

lin-3 gene, mutations in which lead to animals with VPC cell lineage (hence *lin*) defects: all six VPCs behave as if no anchor cell signal were sent, and no vulva is formed. The authors present five lines of evidence for their proposal. First, *lin-3* was cloned and shown to encode an EGF-like growth factor; this would be an appropriate ligand for what had previously been proposed as the receptor for the anchor-cell signal, an EGF-receptor tyrosine kinase produced by the gene *let-23* (ref. 7). Second, a multicopy array of wild-type *lin-3* genes in transgenic animals was shown to induce vulval development by all six VPCs, presumably because of overexpression of the inducing signal by the anchor cell. Third, the vulval overinduction caused by the multiple copies of *lin-3* was suppressed by mutation in *let-23*. Fourth, the overinduction was also suppressed by destruction of the gonad, including the anchor cell. Finally, a *lin-3-lacZ* multicopy transgenic array produced its fusion protein almost exclusively in the anchor cell — and not in the VPCs or surrounding epidermis. These results constitute a firm case for the authors' supposition that *lin-3* produces the signalling molecule in the anchor cell.

Here, as in much recent work, it is

exhilarating to see vertebrate protein motifs turn up in a modest creature where questions of function can be readily addressed. Oncogenic motifs were discovered earlier in the worm from studies of *let-60*, which encodes a Ras protein^{4,8}, and *sem-5*, which has *src* homology regions⁹; both of these genes act downstream of *let-23* in the vulval developmental pathway. Indeed, the worm also uses the products of *lin-3*, *let-23*, *let-60* and *sem-5* in other developmental pathways. Because null alleles of each of these genes result in early larval lethality, the elucidation of the roles of these genes in vulval development depended on the ingenious use of leaky mutations that preferentially affect vulva formation. The use of other alleles and other approaches, perhaps including genetic mosaics, should promote our understanding of what other roles these genes have in development; these would presumably also involve intercellular communication.

The story of vulval development will soon become even richer in molecular detail. Mutations in more than 30 genes result in aberrant vulval development; these genes and their mutations will continue to make wonderful grist for the cloner's mill. Some of the genes have been implicated in the transmission or reception of additional intercellular signals³⁻⁵, one of which is a lateral inhibitory signal between adjacent VPCs; its receptor is probably the product of the well-studied gene *lin-12* (refs 3,5,10), but the signal itself is unknown. Another great unknown is the molecular basis of the beautiful vulval morphogenesis that ultimately results from the sending and receiving of the anchor-cell signal. □

Robert K. Herman is in the Department of Genetics and Cell Biology, University of Minnesota, St Paul, Minnesota 55108, USA.

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High hopes of a trophic factor

R. W. Oppenheim

MOTOR neuron diseases include a number of different sporadic and inherited disorders involving the degeneration or death of motor neurons in the spinal cord and brain¹. Patients suffering from them exhibit progressive muscular weakness and frequently the outcome is fatal. Although there has been progress in establishing the genetic linkage of spinal muscular atrophy², familial amyotrophic lateral sclerosis³ and a rare inherited form of motor neuron disease, X-linked bulbo-spinal-muscular atrophy⁴, there is little reliable information on the aetiology of these diseases and therefore no therapy is available to modify their inexorable and tragic course.

For this reason the paper that appears on page 502 of this issue⁵ is especially significant, as it provides the first real hope that a therapeutic strategy for these devastating diseases may soon be forthcoming. Using a new mouse model of human motor neuron disease, Sendtner and his colleagues have successfully (and dramatically) ameliorated the behavioural and neuropathological course of this disease by the treatment of affected mice with ciliary neurotrophic factor.

Survival

The identification of ciliary neurotrophic factor (CNTF) stemmed from an interest in molecules that support the survival of neurons during development. Neurons in many parts of the developing nervous system, including motor neurons, undergo a process of naturally occurring cell death, whereby a proportion (often one-half or more) of post-mitotic neurons degenerate and die⁶. Historically, it was the discovery of this normal process of cell death in the nervous system that provided the basis for the identification of the first neurotrophic agent, nerve growth factor (NGF), by V. Hamburger, S. Cohen and R. Levi-Montalcini in the 1950s⁷. Nerve growth factor is a target-derived protein that promotes the survival, *in vitro* and *in vivo*, of sensory and sympathetic neurons. A proportion of both types of neuron die naturally during development and the extent of cell loss is thought to be regulated by competition between neurons for limiting amounts of target-derived NGF. In addition, NGF has pharmacological effects on neuronal size and on the synthesis of transmitter enzymes, and promotes survival when neurons have been physically damaged or exposed to toxins⁸.

Because NGF was the first such neurotrophic agent to be identified, it is no surprise that it was also the first to be implicated in the aetiology of a specific

inherited neurodegenerative disorder, familial dysautonomia, which involves loss of sensory and sympathetic neurons. It was proposed that the disease might be caused by a defective NGF gene that leads to the death of neurons because they are deprived of trophic factor. Although the NGF gene has now been excluded as the cause of this disease⁹, there has been growing interest in the possibility that NGF, as well as other trophic agents, is involved in the aetiology of a variety of neurodegenerative disorders¹⁰. Even if not involved in their aetiology, trophic molecules may still be useful as pharmacological therapeutic agents in treating these diseases. A major problem has been that growth factors for neurons that degenerate in diseases such as amyotrophic lateral sclerosis and Alzheimer's and Parkinson's diseases have not yet been identified.

Only recently have molecules other than NGF been found that promote the survival of specific populations of developing neurons. One such is CNTF, a small polypeptide purified from chick ocular tissue and from rat and rabbit sciatic nerve. It rescues avian motor neurons from cell death *in vitro*¹¹ and *in vivo*¹², and also prevents the death of facial motor neurons in neonatal rats following axotomy¹³. Because CNTF is not a muscle-derived molecule, and also does not appear to be expressed (at least in rodents) during the period of naturally occurring motor neuron death, it is still uncertain what role, if any, it plays in normal cell death. Nonetheless, its somewhat surprising effects on motor neurons¹¹⁻¹³ (as indicated by its name, CNTF was thought to be a specific trophic agent whose biological effects were limited to ciliary or parasympathetic neurons) suggested that this protein might be useful as a therapeutic or pharmacological agent. The observation by Sendtner and his colleagues that CNTF can prevent the death of axotomized motor neurons in newborn rats¹³ provided both hope and a sound rationale for this idea, and their paper that appears in this issue carries the analysis to the next logical step, namely testing CNTF in an animal model of human motor neuron disease.

The mouse mutant used, progressive motor neuronopathy (*pmn*)¹⁴, is only the most recent of several genetic animal models of motor neuron disease¹⁵. Compared with two other common mouse models — *wobbler* (*wr*) and *motoneuron degeneration* (*mnd*) — motor neuron degeneration in *pmn* mice appears earlier, progresses more rapidly and the

pathological changes more closely resemble those seen in human motor neuron diseases. Although the gene defect responsible for motor neuron death in *pmn* mice is not known, insufficient or defective expression of CNTF does not seem to be the cause. Normal amounts of biologically active CNTF messenger RNA and protein are present in *pmn* mutants⁵. Furthermore, because motor neurons in *pmn* mutants can be rescued from cell death by treatment with CNTF, it is also unlikely that the genetic defect involves genes necessary for CNTF action, such as those for processing enzymes, receptors or intracellular signalling pathways.

Unperturbed

The fact that CNTF and CNTF-processing pathways are unperturbed in *pmn* is perhaps not surprising, given the lack of evidence that CNTF acts as a biologically relevant factor in motor neuron survival during development. So it is all the more remarkable that a protein that seems not to be involved in the normal survival of motor neurons, and which is apparently not altered in *pmn* mice, can nonetheless have substantial beneficial effects.

Because CNTF has a short half-life, and because *pmn* mice do not tolerate repeated daily injections of CNTF and are too small to accommodate infusion pumps⁵, it was delivered by intraperitoneal injection of a mouse cell line transfected with a genomic construct that releases high quantities of biologically active CNTF. Mutant *pmn* mice treated in this manner lived significantly longer, had improved motor behaviour, exhibited increased survival of facial motor neurons and had increased numbers of myelinated axons in the phrenic nerve⁵. Homozygous *pmn* mice can first be recognized by the appearance of paralytic symptoms on postnatal day 21, at which time considerable motor neuron degeneration may have already occurred. Accordingly, because treatment could only begin at this time, it is especially significant for the human condition that, even when begun at a relatively advanced stage of the disease, CNTF was still highly beneficial.

Despite the dramatic effects of CNTF in *pmn* mice, a number of questions remain. A more systematic and quantitative behavioural analysis of motor performance is needed to determine the extent to which specific motor behaviours are improved. The main behavioural manifestations of the *pmn* mutation presumably involve the altered activity of spinal motor neurons, yet it is not known whether these neurons (as opposed to facial motor neurons) degenerate¹⁴ or, if they do, whether CNTF treatment can ameliorate this

loss. Furthermore, because CNTF receptors are expressed on skeletal muscle¹⁶, it is not yet possible to exclude the possibility that at least some of the effects of CNTF in the *pnn* mice are mediated by muscle. It will also be exceedingly important to determine whether CNTF treatment can continue to be effective beyond postnatal day 50, which was the oldest age examined. Finally, the study of Sendtner *et al.* also does not address the issue of whether corticospinal motor neurons, which degenerate in amyotrophic lateral sclerosis, can be rescued by CNTF.

Nonetheless, it is clear that the effects reported in this paper are of enormous potential significance for the treatment of human motor neuron diseases. It is therefore not surprising that at least two biotechnology companies, Synergen and Regeneron, have already begun preliminary clinical trials of CNTF with patients suffering from amyotrophic lateral sclerosis, or that plans are underway to expand these trials to other forms of motor neuron disease. Because the growth factors that normally promote the survival of motor neurons *in vivo* have not yet been identified, it seems

likely that other molecules remain to be discovered and that they too will have potential as therapeutic agents. □

R. W. Oppenheim is in the Department of Neurobiology and Anatomy, and the Neuroscience Program, Bowman Gray School of Medicine, Wake Forest University, Medical Center Boulevard, Winston-Salem, North Carolina 27157-1010, USA.

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GAMMA-RAY ASTRONOMY

Catching photons from hell

Francis Halzen

THE most energetic γ -rays yet discovered from beyond our Galaxy are described by Punch *et al.* on page 477 of this issue¹. The source, Markarian 421, is a giant elliptical galaxy harbouring an active nucleus. That a distant source like this can be seen at all in teraelectronvolt (TeV = 10^{12} eV) γ -rays implies that its intrinsic luminosity may exceed by 10 orders of magnitude that of sources in our Galaxy such as the Crab supernova remnant.

Fluxes of astronomical high-energy photons are so small that even the latest satellite experiments, such as the EGRET detector on the Compton γ -ray Observatory, cannot extend their observations beyond energies of 10 GeV (10^{10} eV). The key to TeV astronomy and beyond is the use of the Earth's atmosphere as the active detector volume. A mirror with 2° aperture views photon-induced particle showers in the atmosphere over an effective area of 10^5 m². A 0.5 TeV photon will produce several hundred electrons at an altitude of 10 km. Although the shower is absorbed in the air, the Cerenkov radiation produced by the shower particles can be detected with mirrors viewing the sky from mountains during clear, moonless nights.

This Cerenkov method conveniently becomes operative at a threshold not far above the energy at which satellites become insensitive. The real experimental problem is that γ -ray signals are drowned in a background of showers produced by cosmic ray nuclei. Background showers fortunately differ in two essential ways. Most of them will not originate from the direction of the γ -ray source, and they produce hadronic (nuclear) rather than purely electromagnetic showers.

The Whipple Observatory, on Mount Hopkins in Arizona, has over 100 fast photomultipliers to map the image painted by the showers on its 10-m aperture². Pattern recognition techniques are applied to the shower images to reject hadron-initiated events with a minimal depletion of the photon signal. This technique has been fine-tuned in observations of the Crab Nebula. The measured flux (10^{34} erg s⁻¹) has since been confirmed by two French experiments in the Pyrenees, and the Crab is the 'standard candle' for TeV astronomy.

The Whipple telescope was trained on Mk421 from March to June of this year. Although the galaxy is 10^5 times further from us than is the Crab, the count rate

is lower by just a factor of three. A simple consideration of the solid angle subtended by the telescope at the two sources shows that one pays a penalty of 10^{10} in intensity in observing the more distant one. So the Whipple result implies that the TeV flux of Mk421 could be as large as 10^{43} erg s⁻¹, nearly matching the total optical flux (10^{44} erg s⁻¹) from our Galaxy.

The observation is not a complete surprise, and Mk421 was chosen for viewing not without reason. Many theorists have identified 'blazar' galaxies such as Mk421 as powerful cosmic accelerators producing beams of very-high-energy photons and neutrinos. Charged particles are accelerated by shocks in the jets which are a characteristic feature of these radio-loud active galaxies. Many arguments have been given to suggest that protons as well as electrons are accelerated in this way. The γ -rays and neutrinos are the products of decaying pions which appear along the jets as a result of photoproduction by the accelerated protons on ultraviolet photons in the host galaxy. It is remarkable that these sources seem to put out as much energy in TeV γ -rays as in keV X-rays³. Indeed, if we had TeV eyes, the sky would bear an isotropic scattering of bright points, due to these extragalactic objects, with no clear signature of our own Milky Way galaxy.

In selecting a suitable source to look at, the Whipple team turned to the Compton Observatory's catalogue of active galaxies known to emit GeV photons. Although it is hardly the best known example, at a distance of barely over 100 megaparsecs Mk421 is the closest blazar on the list. Stecker, De Jager and Salamon⁴ recently pointed out that TeV γ -rays are efficiently absorbed by infrared starlight (through the process of electron-positron pair production), anticipating that TeV telescopes might have a hard time picking out the brilliant, but relatively distant active galaxy 3C279 through the intergalactic infrared fog. For nearby Mk421, however, the absorption is minimal.

Detailed interest in these observations will not be restricted to γ -ray astronomers. Several neutrino telescopes are being developed — under the sea at Hawaii and in the Mediterranean, in the depths of Lake Baikal, and in the ice cap at the South Pole — which should be able to pick up TeV neutrinos from these extragalactic sources⁵. Already, the specialists will be checking out their

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sums on the back of their proverbial envelopes. And the signs are good. The optical depth (opacity) of a source depends on its magnetic field and is proportional to the energy of the photons.

With a field of 1,000 gauss typically quoted for the jets of active galaxies (although anything from 10^{-4} to 10^4 gauss may in fact be the case), most γ -rays will never escape the source. But the neutrino flux, unaffected by mag-

netic fields, will be enormous. Unattenuated by ambient matter or the intergalactic infrared field, the neutrinos will make their way to our detectors. The clarity of TeV photons from Mk421, six standard deviations above the background, bodes well for detections of these so-called 'neutrinos from hell'. □

Francis Halzen is in the Department of Physics, University of Wisconsin, Madison, Wisconsin 53706, USA.

MARINE POLLUTION

Slick solution for oil spills

David Ollis

WITH 15,000 tonnes of specially coated, hollow glass beads and a little sunlight, the oil slick from the *Exxon Valdez* might have been cleared up within two weeks, according to Heller, Brock and colleagues¹⁻³. Their beads, coated in titanium dioxide, are photocatalytically active and initiate oxidation of organic molecules, such as those in crude oil, spilled on water. Natural microbial action can then finish the task by consuming the more soluble products of the partially oxidized oil.

Each year, a considerable amount of oil is spilled onto the seas: in the 1970s, 4-6 million tonnes of oil entered the oceans annually⁴⁻⁶, of which 6-8% was ascribed to shipping accidents. The groundings of the *Exxon Valdez* (1989, 33,000 tonnes), the *Amoco Cadiz* (1979, 220,000 tonnes) and the *Torrey Canyon* (1967, 100,000 tonnes) exemplify the occasional large contribution from super-tanker accidents to the annual oil-spill mass, but most accidental spills occur during loading and unloading. The traditional methods to cope with these hazardous spills are limited to physical containment and collection using floating booms, application of absorbents such as straw, sawdust, shredded polyurethane or porous volcanic glasses, sinking with

sand, or dispersal by detergents. The large scale of spills that arise from collisions or groundings generates additional logistical problems associated with availability of equipment and personnel, establishment of emergency authority, correct authorization for execution of remedial action, and implementation and execution of a plan of action; these circumstances can so delay the response that a spill becomes a slick covering a hundred or more square miles before substantive action is taken.

The approach of Heller and Brock is to accelerate the natural oxidation of oil caused by solar radiation. Titanium dioxide (best known as the whitener in house paint) is a semiconductor oxide which efficiently absorbs solar near-ultraviolet radiation (300-400 nm) and which is activated by this radiation for catalytic oxidation. It is also denser than water or oil, which is why Heller, Brock and colleagues immobilize it on hollow glass beads. The authors have settled on hollow beads 100 μ m in diameter, coated with 300-1,000-nm particles of titanium dioxide and further treated with alkyl siloxanes to make the beads hydrophobic so they will attach to oil surfaces and shun water when initially applied.

Under continuous activation by light,

the titanium dioxide particles produce hydroxyl and hydroperoxyl radicals by the oxidation of hydroxide ions in water or reduction of molecular oxygen from air. These chemical oxidation agents attack oil, including alkanes, alkenes and aromatics, to give products with hydroxyl, carbonyl and carboxylate functionalities. The increased solubilities of these products, the authors argue, would allow accelerated dissolution of the slick, thus providing a more rapid exposure to the indigenous degrading microorganisms in sea water.

The limiting rate at which coated beads can attack oil slicks is given by the flux of useful solar radiation (about 2 per cent of the sea-level flux) which can be scavenged by the photocatalyst; this is equivalent to about 10^{15} photons per second per square centimetre, or roughly a molecular monolayer per second. If the quantum yield were unity, then every photon would oxidize one molecule of hydrocarbon. For an average molecular chain length of 1 nm, a film 100 μ m thick could be converted in about 1 day; for a quantum efficiency of 10%, 10 days would be required. Heller and Brock reported¹ a quantum yield of 5% for catalyst-coated beads on 2-octanol when palladium was added to the catalyst; this efficiency would require about 20 days for complete conversion of a 100 μ m film, as might typify an oil slick. (Such a use of palladium may be prohibitively expensive on a practical scale.)

An oil spill is always moving and changing composition and dimensions. An early estimate⁴ assigned the environmental fate of the spilled oil mass as follows: evaporation, 25%; dissolution, 5%; photochemical oxidation, 5%; biodegradation, 30%; disintegration and sinking, 15%; and residue, 20%. An estimate⁷ for the *Exxon Valdez* spill contained some similar figures but also new categories: evaporated, 35%; recovered, 17%; burned, 8%; biodegraded, 5%; dispersed, 5%; oil slick, 10%; and shore deposit, 18%. The spread-out oil slick is the best candidate for photocata-

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Greenpeace/Merjenburgh

Cleaning up with floating booms after the oil tanker *Exxon Valdez* ran aground in Prince William Sound, Alaska, on 24 March 1989.

lytic clean-up; however, this thin film form is also already most amenable to direct photooxidation and biodegradation at an appreciable rate⁷.

The actual rate of photoassisted biodegradation will depend on many variables: quantum efficiency of catalyst, dissolution rates (a function of wind and wave motion), and the local microbial population of hydrocarbon and oxidized hydrocarbon degraders. The biodegradation rate itself will vary with temperature, dissolved oxygen and nutrient (nitrogen, phosphorus) levels. Heller, Brock and colleagues argue that, with a 10% quantum yield for the photocatalyst, a tonne of coated beads could convert a tonne of oil spread out to a 100 μm film within two weeks. The *Exxon Valdez* spill reached an area of 100 square miles in three days, producing a film of just such a treatable thickness.

Uncertainties abound with any proposed technology. The *Exxon Valdez* spill area increased to 500 square miles, owing to rough weather on the fourth day; high waves and large-scale mixing create 'chocolate mousse' emulsions which would hide beads from solar radiation. The application of light-absorbing beads will also slow the homogeneous photochemistry which can photolyse the most biodegradation-resistant hydrocarbon bonds⁶. Although titanium dioxide is inert, stable and so pretty benign, it is fair to be nervous about the environmental effects of vast quantities of the coated beads. The oil spill itself, as well as the partial oxidation byproducts, if present in sufficient dissolved levels, may be toxic or inhibitory to the degrading microbes, or may interfere with other marine life by disrupting, for example, chemoreceptor-dependent biological activity (feeding, mating) of some marine organisms⁶.

Nonetheless, for accelerated removal of oil slicks and perhaps oil spills, this new treatment concept which relies on sunlight, air, and a benign mineral, titanium dioxide, that is virtually insoluble in water certainly deserves further consideration, including field testing at larger scales. $\square$

David Ollis is in the Department of Chemical Engineering, North Carolina State University, Raleigh, North Carolina 27695, USA.

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Does the mid-crust flow?

Wayne Thatcher

THE Earth's crust is brittle, and its mantle ductile, but what happens between? Measurements of surface movement following the injection of magma into fissures near Krafla volcano in Iceland, reported by Foulger *et al.* on page 488 of this issue¹, suggest that the mid-to-lower crust, perhaps 8–30 km deep, deforms plastically, like the mantle.

The depth in the lithosphere to which faulting extends is poorly known. This parameter determines how much of the 100-km-thick continental lithosphere participates in the heterogeneous processes of faulting and folding seen at the Earth's surface and what portion is more homogeneous and flows in a quasi-continuous ductile manner. The issue depends on the rheological layering of the lithosphere, the way in which rocks at a given depth range deform in response to body forces and applied stresses. At least in the upper 10–15 km of the Earth's crust, strain is proportional to stress and rocks deform elastically or fracture in a brittle manner, a consequence of the relatively low ambient temperatures in the upper crust and the composition of the crustal rocks, largely the minerals quartz and feldspar.

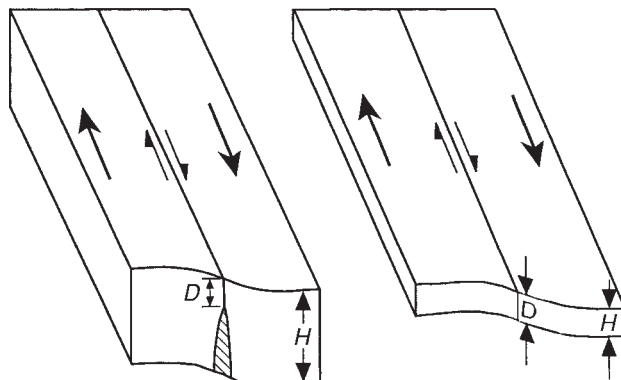
Upper mantle

In the upper mantle, below 30–50 km depth, where olivine is the dominant mineral and temperatures exceed 800 °C, most of the deformation of the lithosphere is believed to occur by ductile flow. Between the brittle/elastic upper crust and the viscously deforming mantle the rheological behaviour is poorly known, but if temperatures are high enough there may be a rapid transition to ductile flow. Disciplines as diverse as structural geology², rock mechanics³, elastic-wave seismology⁴ and geothermal studies⁵ have been applied to constrain the rheological properties of crust and mantle rocks.

Geodesy, as used by Foulger *et al.*, helps because motions at depth exert shear tractions on the brittle crust that produce measurable deformation at the Earth's surface. An important way in which this happens is when episodic events in the brittle part of the lithosphere (earthquakes and volcanic eruptions) cause stress changes and consequent transient deformation in the rocks lying beneath. Studies of the cyclic build-up and release of earthquake-related crustal strain at major plate boundaries have identified post-earthquake transient movements and related them to the mechanical properties of the subseismogenic lithosphere⁶. The report by Foulger *et al.* provides evidence for a similar process following dike intrusion events in the exposed spreading rift zone of the Mid-Atlantic Ridge in Iceland.

The cyclic accumulation and release of stress occurs because the steady movement of the large lithospheric plates is retarded near their boundaries by frictional resistance. Once these steady motions build up stresses sufficient to overcome friction, abrupt relative motion occurs, as earthquake fault-slip across transcurrent and compressional boundaries, and as dike intrusion on the mid-ocean ridges. These events in the brittle crust transfer stress to the region beneath, which responds either by aseismic slip or separation within a narrow shear zone, or by bulk flow over a broader region. Elsasser's perceptive paper⁷ outlined these basic principles during the late 1960s when the plate tectonic model was first introduced. However, realistic models that accurately determine the crustal movements throughout the earthquake deformation cycle have been developed only since the late 1970s⁸, and data comparisons are often limited by geodetic measurements that sample only restricted portions of the cycle with incomplete spatial coverage.

Two contrasting models for the cyclic accumulation and release of earthquake related crustal straining. In a thick elastic lithosphere (left), ductile flow in the mid- or lower crust is negligible at earthquake-repeat timescales, but with a thin elastic lithosphere (right), ductile flow controls the transient postseismic and steady inter-event deformations. *D*, Depth of earthquake slip; *H*, thickness of brittle/elastic upper crust.



RÉSUMÉ

Furthermore, many of the data suffer from a fundamental ambiguity of interpretation, as there are two contrasting rheological models which can generate identical or nearly identical motions at the Earth's surface⁶ (see figure). In the first, the thick-elastic-lithosphere model, the depth of fault slippage, D , is much less than the thickness, H , of the elastic lithosphere, and transient and steady inter-event motions are caused by episodic and uniform aseismic fault slip beneath the rupture plane. In contrast, for the thin-elastic-lithosphere model, $D \approx H$ and it is transient and steady ductile flow in the viscoelastic substrate that generates the inter-event movement patterns. This ambiguity is not always acknowledged or fully appreciated in the published literature, and individual researchers often employ one model without rationalizing their choice.

The general problem here is the absence of independent constraints on mid- and lower-crustal rheology appropriate to the timescales of event recurrence (usually hundreds to thousands of years). In this regard the Icelandic data set may have a distinct advantage. Electrical resistivity measurements quoted by Foulger *et al.* suggest a ductile rheology for the mid-to-lower crust, and effective viscosities inferred from post-rifting surface movements during 1987–90 are broadly consistent with those based on modelling by post-glacial rebound in Iceland. Nonetheless, doubts remain, as the depth resolution of surface measurements is generally crude, and it seems entirely possible that a model including post-event aseismic dike opening in the mid-crust could reproduce the observed displacement field and yet not significantly violate the other geophysical constraints.

Cycles

Of course the mid-crust could also flow at timescales longer than those which are directly relevant to seismic or volcanic eruption cycles. However, the potential merit of geodetic observations is to constrain the minimum value of the effective viscosity of the mid-crust. If post-event relaxation occurs on timescales of decades to centuries, and if this relaxation were due to crustal flow, then effective viscosities would be less than about 10^{20} Pa s, a value sufficiently low to decouple the tectonics of the brittle upper crust from the steady flow of the ductile lower lithosphere.

Another common limitation of inter-event deformation data is the difficulty of clearly distinguishing post-event transient movements from steady (uniform rate) long-term motions. For example, the Icelandic data cover only a short, 3-year interval about 10 years after the rifting episode which is alleged (reason-

ably) to be responsible for the observed high rates of post-rift transient deformation. However, there are as yet no data demonstrating either a temporal decline in strain rate or the expected migration of the strain rate field. Indeed, but for a small number of relatively complete post-earthquake geodetic data sets from Japan, the evolution of postseismic transient deformation is poorly documented, and even for the best constrained of the Japanese case studies there is room for several alternative views⁹.

Declines

The general problem is that although there is convincing evidence for short-term (1 year or less) postseismic transient movements which are plausibly related to accelerated aseismic slip immediately down-dip of the earthquake rupture plane, supporting evidence for declines in strain rate over decades or longer is more elusive, and it is these latter movements that may indicate ductile flow in the mid- or lower crust. Again, the Icelandic data appear to have a distinct advantage, as the maximum relative displacement rate during 1987–90, about 40 mm yr^{-1} , looks as though it significantly exceeds the steady-state spreading rate of 19 mm yr^{-1} across the Mid-Atlantic Ridge in Iceland, supporting the authors' interpretation of their data in terms of transient motion.

These results point to clear ways in which new geodetic measurements can contribute to improving understanding of lithospheric rheology and the processes occurring beneath the brittle upper crust. Careful tracking of the spatial and temporal evolution of the strain-rate field following major earthquakes and episodes of dike injection would be especially valuable. The versatility and relative ease of surveying with the satellite-based Global Positioning System¹⁰ makes it well-suited for this purpose, both through the establishment of new geodetic networks in the source region of major events and through resurvey of existing nets, which in many active regions date back 100 years or more. □

Wayne Thatcher is currently in the Department of Earth Sciences, University of Oxford, OX1 3PR, UK.

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Paternity pursuit

ENGAGING in the astronomical equivalent of an identity parade, P. J. Mouginitis-Mark *et al.* have tried to track down the parentage of the SNC family of meteorites, comprising shergottites, nakhlites and Chassigny (*J. geophys. Res.* **97**, 10213–10225; 1992). The consensus is that the SNC meteorites are all chips off the martian block, probably ablated in a single planetary impact. But could the scar left behind be recognized? From the meteorites' petrology, the authors narrowed the search to the Tharsis volcanic region of Mars. Using mug shots of craters from the Viking mission, they found nine likely culprits, each of which is young (less than 180 million years old) and large enough (over 10 km across) to supply material that is still arriving at Earth. But none fully fits the description, and the authors are, as they say, continuing with their enquiries.

Strong stuff

THE marine worm, *Phragmatopoma*, ranks high among nature's builders for the economy and ingenuity with which it constructs its communal labyrinth, made of what appears to be masonry. J. H. Waite *et al.* (*Biochemistry* **31**, 5733–5738; 1992) have isolated the precursors — the unset cement — of one component of the composite (particles of debris caught from the circulating water make the other). Two proteins are abundant in the creature's thorax, each characterized by a repeating amino-acid motif in which 3,4-dihydroxyphenylalanine is prominent. This catechol is enzymically converted into quinone, which crosslinks the protein chains and probably attaches them to the substratum. The mortar outclasses man-made counterparts in that it bonds to unprepared and wet surfaces, is not choosy about the filler material, and functions at low mortar/filler ratios. Not bad for a mere worm.

Mirror Image

A NEW twist is given to Berry's phase by researchers at Caltech (M. Segev *et al.* *Phys. Rev. Lett.* **69**, 590–592; 1992). The quantum record of a particle's history, Berry's phase is a phase angle which changes as the particle's environment is altered. Once the environment is brought back to its original state, the phase can be measured by comparison with a reference particle; in practice this is typically done interferometrically using beams of photons or neutrons. The usual picture of the phase rotating is made manifest in the new work, in which the image of a cross is passed through a many-mirrored periscope. The emerging image it turns out, is rotated by an angle equal to the sum of the phases acquired on each reflection.

Something completely different

Henry Gee

THE description in 1892 of a fossil shrimp would rank as one of this year's most obscure centenaries, were it not for the fact that the fossil came from the 540-million-year-old Middle Cambrian Burgess Shales of British Columbia and was named *Anomalocaris canadensis*. Ninety years later, Harry Whittington of the University of Cambridge and Derek Briggs, now at the University of Bristol, redescribed it not as the body of a crustacean, but as the claw of an altogether larger, stranger animal. By that time the animals from the Burgess Shales had become bywords for the unusual, and were taken out for a centennial airing at a recent meeting*.

Are the Burgess creatures really as weird as they first appear? In his best-selling book *Wonderful Life*, Stephen Jay Gould contends that the strangeness is real, that early life was manifested in a far greater variety of 'disparate' body plans than exists today. But Briggs, together with Bristol colleague Matthew Wills, and Richard Fortey of the Natural History Museum in London suggest that this disparity is illusory: modern crustaceans alone exhibit a morphological variety at least as impressive as all the Burgess Shales' arthropods put together (*Science* **256**, 1670–1673; 1992). The argument centres on how one defines higher taxa, something that proves extremely hard when confronted with the mess of early- and mid-Cambrian forms in the first flush of adaptive radiation, rather than with the essentially static modern biota upon which taxonomic groups are based. Is the problem simply semantic, or was there something special about the tempo and mode of evolution in the Cambrian?

The problems have been ameliorated somewhat by the discovery of other well-preserved Cambrian faunas, which can be used to set the Burgess Shales in context. Most notable are the Chengjiang fauna of China and the Lower Cambrian Sirius Passet fauna from Greenland (John Peel, Geological Survey of Greenland, Copenhagen; Graham Budd, Cambridge). New additions continue to be discovered, such as the Emu Bay Shales from South Australia (Christopher Nedin, University of Adelaide). These faunas serve to fill in some of the gaps. For example, *Anomalocaris* and its

relatives, which now include that other Burgess oddity *Opabinia*, formed a diverse and widespread group which, although peculiar, might be fitted into the metazoan family tree somewhere between the onychophorans and the arthropods proper (the precise position is contentious). Amazingly, the first complete specimen of *A. canadensis* was

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

A 'shoal' of the Burgess Shales fossil *Eldonia ludwigi*

found only last year, and the two species from the Burgess (the other is *A. nathorsti*) may soon be joined by a third form (Des Collins, Royal Ontario Museum, Toronto).

The onychophorans, represented today by the velvet worm *Peripatus* of tropical forests and its relatives, underwent an impressive radiation in the Cambrian, and several forms are now thought to belong to this group (see for example, L. Ramsköld and Hou Xian-guang, *Nature* **351**, 225–227; 1991, and references therein). Even *Hallucigenia*, the *sine qua non* of Burgess oddities, seems to have been among their number. Many Burgess animals were indeed very strange, but not so strange as to have been completely unrelated to extant metazoans.

The same may not be true for the Ediacaran faunas (an earlier flush of arguably aberrant macroscopic creatures, from the latest Precambrian), but

some Ediacaran forms seem to have survived the end of the Precambrian. An animal from the Burgess Shales resembles an Ediacaran form similar to modern pennatulaceans, or sea-pens, relatives of corals (Simon Conway Morris, Cambridge). That this connection seems remarkable reflects our ignorance of modern as well as fossil forms, particularly those from the deep sea. A more egregious example is that of *Eldonia* (see figure), a disc-shaped fossil that largely defied interpretation until an examination of hundreds of specimens by Duncan Friend (Cambridge) revealed detailed similarities with a group of deep-sea pelagic holothurians (sea cucumbers).

These correspondences might seem reassuring, given that it may be impossible in principle to classify a fossil creature so aberrant that it cannot be interpreted in the light of extant forms (Colin Patterson, Natural History Museum, London), a problem that seemed particularly acute for the Burgess Shales until other faunas were discovered, and remains pressing for most of the Ediacara fossils.

Nevertheless, much recent circumstantial evidence seems to indicate that the Cambrian 'explosion' itself, irrespective of the pedigrees of its *dramatis personae*, was a 'real' event, in that early metazoans were prey to a combination of evolutionary circumstances that occurred only once in the history of life. First, morphological diversity in some groups seems to have preceded taxonomic diversity: a plethora of taxa, each with a distinct body plan, was replaced by a few taxa, each of which became relatively speciose.

Although Gould may have pressed the point rather far, morphological 'disparity' could have been greater than expected, at least in particular cases such as the blastozoan echinoderms (Michael Foote, University of Michigan, Ann Arbor).

Second, the degree of endemism seems to have been particularly high. High endemism goes with high extinction rates, and combined with a respectable amount of morphological disparity within and between certain groups, mass extinctions in the Middle Cambrian were at least as catastrophic as the end-Permian extinction that wiped out more than 90 per cent of marine genera 250 million years ago (Philip Signor, University of California, Davis).

For those deflated by the slow extirpation of the mystery that makes the lives and times of Cambrian creatures so appealing, the Cambrian still holds some powerful enigmas. The trace fossil *Cli-*

* Fifth North American Paleontological Convention, Field Museum of Natural History, Chicago, 28 June–1 July 1992.

mactichnites was described as long ago as 1860, and has been found in Late Cambrian sediments in North America. The fossils are trails that look rather like tyre tracks, up to 20 cm wide. Recent work suggests that the tracks could have been formed only on damp, subaerially exposed sand, such as that found on tidal flats (Ellis Yochelson and M. Parrish, National Museum of Natural History, Washington DC, with Michael A.

Fedonkin of the Palaeontological Institute, Moscow). The fossils indicate the presence of a large animal that crawled onto land more than 150 million years before the amphibians. But despite valiant attempts at reconstruction, nobody has any idea what this animal looked like. □

Henry Gee is an assistant editor of *Nature*.

NEUROBIOLOGY

Modules for molecules

Gordon M. Shepherd

THE identification of candidate membrane receptors for odour molecules¹ has generated a huge amount of interest in how the molecular information is processed in the nervous system². The first synaptic relay in the olfactory pathway occurs in modular structures called glomeruli, which are present in both vertebrates and invertebrates, implying that they have a central part in organizing neural space to encode molecular information. The time has arrived for neurobiologists to take glomeruli seriously, hence the recent meeting* devoted to these enigmatic structures.

Some rules of connectivity can be specified. In all species, the sensory neurons project directly, with no branches, to their target glomerulus. In vertebrates, the axons within a glomerulus branch only sparingly, making 6–12 synapses on the dendrites of relay neurons (mitral or tufted cells) and interneurons (periglomerular cells) (C. Greer, Yale University). Synaptic connections among the dendrites form a dense concentration of excitatory and inhibitory microcircuits where the first processing of olfactory information takes place. In invertebrates, the sensory terminals end primarily on interneurons, with complex dendro-dendritic feed-forward connections to the projection neuron dendrites (D. Malun, University of Regensburg; DeF. Mellon, University of Virginia).

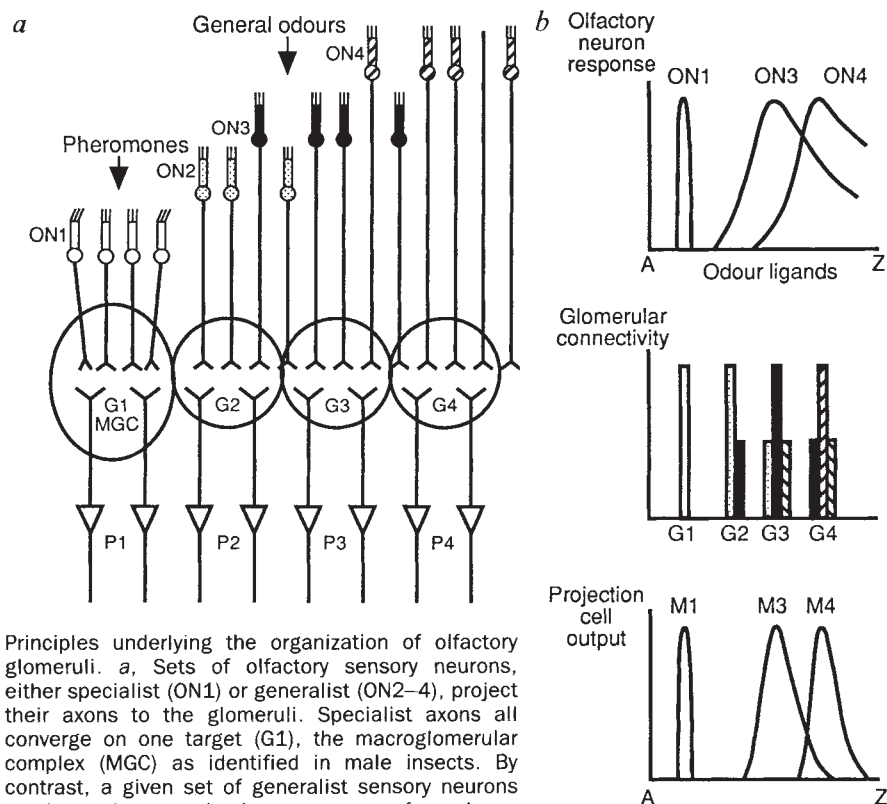
The developmental mechanisms for assembling this complicated apparatus centre on the sensory axons, and critical interactions with glial cells have been demonstrated in some species (G. Burd and L. Tolbert, University of Arizona). In vertebrates, the glomerular sheet is at first relatively undifferentiated; distinct glomeruli begin to appear in the first postnatal week (rat), and the adult pattern emerges by weaning at 3 weeks. The numbers stabilize thereafter (E. Meisami, University of Illinois) —

although confocal microscopic observations of olfactory bulbs stained with supravital dyes suggest that some new glomeruli continue to be added³. Vertebrate sensory neurons turn over throughout adult life: how their axons find appropriate glomerular targets is a challenging question for developmental

biologists.

Considerable progress was reported on analysing glomerular physiology. Mitral cells in a local region of the rabbit olfactory bulb characteristically respond relatively narrowly to 2–3 structurally similar members of the same class of odour compounds, for example C2–C12 aliphatic acids (K. Mori, Osaka Bioscience Institute). Structure–response analyses have also been carried out in cockroach antennal cells (J. Boeckh, University of Regensburg). These results support the idea that glomeruli gather input from neurons bearing receptor proteins with similarly broad molecular affinities (R. Reed, Johns Hopkins University), and that one function of the glomerulus is to sharpen the tuning, performing a kind of feature extraction on properties of the stimulating molecules (see figure).

Some properties of the response are surprisingly similar in vertebrates and invertebrates. A prominent response pattern of both mitral cells and antennal lobe projection neurons to odour pulses



* The Glomerulus, Tegernsee, Germany, 9–11 June 1992.

consists of an initial brief hyperpolarization, followed by a depolarizing-hyperpolarizing sequence (J. Kauer, Tufts University; J. Hildebrand, University of Arizona). These complex patterns now are open to correlation with the specific synaptic interactions being revealed in the glomerular microcircuits (J. Scott, Emory University; H. Hatt, Technical University of Munich). Their similarity suggests that, despite the differences in outward morphology of the vertebrate and invertebrate neurons, the processing steps through the glomerular circuits may be fundamentally much the same. This seems to be one of the clearest cases of equivalence between vertebrate and invertebrate nervous mechanisms at the systems level.

Of special interest is the insect macroglomerular complex (MGC), a cluster of identifiable glomeruli specific to the male, which receives input from sensory neurons narrowly tuned to components of female sex pheromones^{4,5}. Projection neurons connected exclusively to the MGC also respond specifically to such components, implying that the MGC acts as a labelled line for these signal molecules (see figure). Vertebrate examples of identified glomeruli include the accessory olfactory bulb, specialized for mating odours, and the modified glomerular complex, active in suckling rat pups.

Glomeruli lend themselves well to quantitative analysis. In different species they vary in size from 20 to 200 µm in diameter. The numbers of glomeruli are known for several insect species, varying between about 50 and 300 (J. Rospars, INRA-Versailles; J. Boeckh; J. Hildebrand), and many glomeruli can be individually identified. Inputs converge from 100,000–300,000 sensory neurons and outputs emerge through 100–800 projection neurons. In the cockroach MGC, 70,000 pheromone-specific sensory neurons converge onto only 15 projection neurons, presumably contributing to the extraordinary sensitivity of the male moth to the female pheromone (J. Boeckh).

An active frontier in glomerular research is the use of noninvasive markers to reveal spatial activity patterns. The 2-deoxyglucose mapping method has shown that different odour stimuli elicit overlapping but different patterns of active glomeruli, and that individual glomeruli appear to be functional units in these patterns⁶. Mapping of the distribution of *c-fos* messenger RNA during odour stimulation has provided strong supporting evidence for such patterns (M. Leon, University of California, Irvine). Voltage-sensitive dyes are revealing the complex temporal evolution of glomerular patterns elicited by odour stimulation in the salamander (J.

Kauer), and comparisons of such results with patterns revealed by these methods in other sensory systems are under way.

The relation of patterns of glomerular activity to olfactory behaviour generated considerable discussion. The fact that much of the glomerular sheet can be ablated in rats without apparent effect on odour detection (R. Hudson and H. Distel, University of Munich) may have several explanations. One is the representation of some degree of responsiveness to a given odour in many glomeruli. Another is a great deal of plasticity in the system; in rat pups, after ablation of the normal area of focal 2-deoxyglucose glomerular activity induced by peppermint odour, a new site appears (M. Leon), implying that new connections develop rapidly. More sensitive behavioural tests will be needed, particularly for the ability to discriminate between several odours.

A remarkable unanimity on a number of key points emerged from the meeting. The glomerulus receives convergent input from sensory neurons with similar receptor proteins (almost certainly for the insect macroglomerulus, probably for ordinary glomeruli), thus constituting a complex labelled line. It is a site of intensive information-processing, extracting and combining features of the stimulating molecules. The relative positions of glomeruli have no special importance, but rather reflect relationships between the molecular information being processed. And the projection neurons resemble retinal ganglion neurons in carrying a distributed representation of the parallel processing that has taken place through the glomerular population.

All of these properties are starting points for future experiments, and there can be few more promising subjects for fruitful cooperation between vertebrate and invertebrate neurobiologists on principles of neural organization. That those principles may tell us why information contained in different signal molecules is universally mapped into neural space by means of modules called glomeruli is not the least of the attractions. □

Gordon M. Shepherd is in the Section of Neurobiology, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA.

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Horn of plenty

THE African black rhinoceros is rapidly being hunted to extinction. The luckless animal carries on its nose a pair of horns which are much prized in the Orient for their (imaginary) medicinal and aphrodisiac powers. In a heroic effort to save the species, a team of biologists is capturing as many rhinos as possible, and sawing their horns off. This destroys their value to the hunters, at least until the horn has grown again. Daedalus planned to saturate the market with fake rhino horn (perhaps from cow horns), but is now developing a more honest strategy.

He points out that the horn is essentially an outgrowth of the skin, like a hair or a fingernail. So instead of just being sawn off, it could be excised as a viable structure, just as hair-transplant surgeons take viable hair roots from the back of a bald patient's head, and reimplant them on his denuded scalp. A rhino whose horns had been excised would presumably never grow new ones. Thereafter, it could live and breed happily without fear of the poachers.

The excised horn, says Daedalus, could also survive. He toyed with the appealing notion of reimplanting it on the nose of a horse, thus creating a genuine unicorn, but the tissue rejection problems would be acute. It would be easier to root the horn into an artificial tissue-culture substrate, fed from a heart–lung machine with oxygenated rhino blood of the correct blood-group and enriched with appropriate nutrients. The horn-base cells would be unaware that they were no longer part of a living animal. The excised horn would continue to grow.

Daedalus reckons that it should last for ever. With luck it will be free of the 'programmed cell death' that afflicts many living tissues — after all, human hair and nails are said to keep growing for some while after their owner has died. But even a limited-life horn might be 'immortalized' by some sort of hybridoma technology, perhaps by infusing it with the appropriate proteins from a papilloma virus. It could also be supplied with suitable hormones to maximize its growth rate. Even a continuous pull might help — human hair grows faster if pulled steadily.

Thus the absurd demand for rhino horn could be satisfied without endangering the species. The noble serried ranks of cultured rhino horns in DREADCO's horn farm will effortlessly supply the market. Growing far faster than on their original sites, the horns will be cropped repeatedly, either for powdering or direct export as authentic horn sections. DREADCO biologists will be curious to discover whether, against all the odds, they have any useful medicinal properties. David Jones

Coelacanth fins and evolution

SIR — Major developmental mutations, such as the well-known homeotic mutations in the fruitfly *Drosophila*, can readily be induced in the laboratory, but are almost invariably deleterious and are widely regarded as unimportant to evolution¹. However, the median fins of coelacanth fishes seem to furnish evidence of a dramatic developmental mutation event, comparable to homeotic transformation, in the early history of the group.

The paired fins of coelacanths (such as the recent *Latimeria*), lungfishes and other lobe-finned fishes (sarcopterygii) are structurally comparable to the limbs of tetrapods and appear to develop in a similar way². The dorsal and anal fins are usually quite different, their internal skeletons taking a form similar to *a* in the figure. Phylogenetic evidence³ indicates that the primitive type of median fin skeleton in jawed fishes is a row of separate, parallel rods; *a* in the figure is essentially a 'condensed' version of this, with the rods supported by a single basal plate. Modern lungfishes have reverted to the primitive pattern⁴. The paired fins of sarcopterygians are thus of a unique, limb-like construction, whereas the median fins in most members of the group seem to be broadly similar to those of other fishes. This is not the case in coelacanths.

The posterior dorsal and anal fins of coelacanths are strongly pedunculate (*b* in the figure). The internal skeletons, musculature and innervation of these fins correspond extremely closely to those of the paired fins⁵. Further, they

are supported by complex basal plates that do not resemble those of other lobe-finned fishes but are closely comparable in structure and ossification pattern to the coelacanth pelvis (*d-f* in the figure). The anterior dorsal fin, by contrast, is of a generalized sarcopterygian type. Film of swimming *Latimeria* shows that the posterior dorsal and anal fins move in sequence with the paired fins, whereas the anterior dorsal fin does not.

The posterior dorsal and anal fins of coelacanths are fundamentally different from those of other sarcopterygians, but resemble coelacanth pelvic fins very closely. If this degree of similarity obtained between (for example) pelvic fins of two taxa, rather than between paired and median fins of the same animal, it would unquestionably be accepted as evidence of homology. The most obvious interpretation of the pattern, in the absence of direct embryological evidence, is that paired-fin structures are being expressed at the posterior dorsal and anal fin sites. This inference suggests another interesting possibility. Although coelacanths have possessed dorsal and anal fins of apparently modern pattern since the Devonian⁶, it seems clear that they are derived from fishes with 'normal' median fins. The differences between the two fin types are so great, however, that it is very difficult to imagine what structural intermediates would be like or how they could function. It is easier to envisage the replacement as a single-step event caused by a simple 'switch' in gene expression. A major aspect of coela-

canth morphology may thus have arisen quite suddenly, rather than through gradual change.

Our understanding of coelacanth development and genetics is as yet too limited to test the ideas presented here. The inferred sudden change from one median fin structure to another could be falsified by evidence from the fossil record. The Upper Devonian genus *Miguashaia*, the probable sister group of all other coelacanths⁶, appears to have 'normal' sarcopterygian median fins; further work on Devonian coelacanths may thus help to cast light on this interesting problem.

P. E. Ahlberg

Department of Zoology and University Museum,
University of Oxford,
South Parks Road,
Oxford OX1 3PS, UK

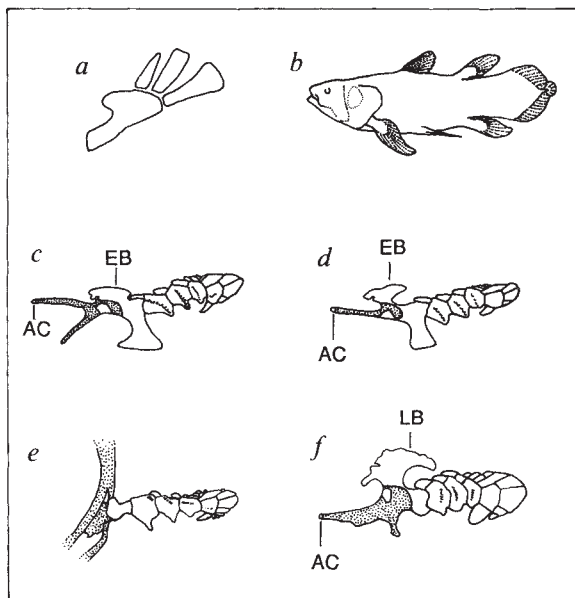
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Westhof's rule

SIR — Associations via hydrogen-bonded base pairs between different polynucleotide chains or between different segments of a polynucleotide chain lead to the various polymorphs of antiparallel DNA structures but also to unusual DNA structures such as triplexes, quadruplexes¹ or parallel helices², and also underlie the three-dimensional folding of RNA molecules. To specify a structure, one needs to indicate the nature of the hydrogen-bonding sites (Watson–Crick or Hoogsteen atomic positions); the orientation of the base with respect to the sugar (*anti* or *syn*)³; the position of the glycosyl bonds with respect to each other (*cis* or *trans*, that is, both on the same side or on different sides of the base pair); and finally, the relative orientation of the polynucleotide chains (antiparallel or parallel). Thus, the canonical antiparallel B-DNA structure possesses base pairs involving only the Watson–Crick sites with an *anti* conformation for the bases and a *cis* position of the glycosyl bonds.

I wish to suggest a general rule relating the various stereochemical constraints that apply to pairings involving at least two hydrogen bonds between any type of nucleotide of usual chirality. Less general statements have already

a, Posterior dorsal fin support of the osteolepiform *Eusthenopteron* in left lateral view (from ref. 7); *b*, sketch of *Latimeria chalumnae*; *c–f*, median fin supports of *Latimeria*, ossified parts stippled, lepidotrichia not shown. *c*, Posterior dorsal fin in left lateral view; *d*, anal fin in right lateral view, inverted; *e*, right pectoral fin in internal view, ends of shoulder girdle omitted; *f*, right pelvic fin and pelvic moiety in dorsal view (*b–f* modified from ref. 5). Note the small 'anterior cartilages' (AC) capping the anterior ends of the pelvis and median fin basal plates, and the similar ossification patterns. The 'external blades' (EB) of the dorsal and anal fin supports are double structures facing left and right, unlike the single 'lateral blade' (LB) of the pelvis. The lateral blade is deflected dorsally; if a pelvic moiety was mirrored through the sagittal plane of symmetry in the same way as the dorsal and anal fin supports, the lateral blade and its mirror image would correspond perfectly to the 'external blades'.



PREDICTION TABLE FOR RELATIVE CHAIN DIRECTIONS IN NUCLEIC ACIDS

PREDICTOR MATRIX FOR ALL TYPE OF NUCLEIC ACIDS			
Watson—Crick vs Hoogsteen H-bond sites	<i>Anti</i> vs <i>syn</i> glycosyl bonds	Position of base pair glycosyls	
		<i>Cis</i>	<i>Trans</i>
Chains are			
Same	Same	Antiparallel	Parallel
Same	Different	Parallel	Antiparallel
Different	Same	Parallel	Antiparallel
Different	Different	Antiparallel	Parallel

been made by Sundquist and Klug⁴. If the two paired bases (1) hydrogen-bond via the same sites (giving either Watson-Crick . . . Watson-Crick or Hoogsteen . . . Hoogsteen pairings) and (2) adopt the same conformation of the base with respect to the sugar (either *anti* or *syn*), then a *cis* position of the glycosyl bond leads to antiparallel chains and a *trans* position to parallel chains. Each time one of the two starting conditions is not met, a reversal in the orientation of the chains is obtained (see table). In the transfer RNA structure³, for example, the Hoogsteen . . . Hoogsteen base pairing A9-A23 presents a *trans* position of the glycosyl bonds with *anti* conformations and a local parallel orientation of the strands, whereas the Watson-Crick . . . Hoogsteen pairing U8-A14 presents the same characteristics but with a local antiparallel orientation of the strands.

The above rules apply to the nucleotides which are pairing; additional information or assumptions are required for assessing the links between the different levels of base pairs and the chirality of the structure. They are, however, useful for generating and selecting models to test against observations from X-ray, NMR or chemical probe techniques. Further, these rules could be formalized for developing computer software able to generate automatically three-dimensional models of nucleic acids.

Because of the dyad, the fully symmetric *trans* self-pairs (for examples U . . . U or A . . . A) always have parallel chains as long as the nucleotides obey 2-fold symmetry, thus adopting the same glycosyl conformation. Pairings involving N2/N3 of guanines or N3 of adenines are excluded. A referee of this Scientific Correspondence has suggested

the following compact expression of my rule: assign a parity of +1/-1 for (1) pairing via the same/different sites; (2) same/different glycosyl bond conformations; (3) same/different positioning of base-pair glycosyls. Then, if the product of these three parity numbers is positive, the polynucleotide chains will be antiparallel (the usual orientation) and, if the parity product is negative, the chains will be parallel.

Eric Westhof

*Institut de Biologie Moléculaire
et Cellulaire,*

CNRS, F-67084 Strasbourg Cédex, France

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Cometary joke

SIR — Daedalus's mechanism¹ of "exploding comets" through the condensation of solid hydrogen shows imagination but a poor knowledge of the literature. It is flawed for reasons well known to astronomers for many years.

Long-period comets from the Oort cloud making their first pass through the planetary region are seen to be anomalously bright on their inbound leg. This had previously been attributed to a 'frosting' of interstellar molecules condensed on the cold nuclei during their long residence in the Oort cloud at distances of 2×10^4 AU or more^{2,3}, much like Daedalus's solid hydrogen coating. But the cometary nuclei are still exposed to ultraviolet and X-ray photons in deep space⁴, as well as to the intense flux of galactic cosmic rays⁵. These will act to modify the chemistry of the frosting, sputtering off the more volatile materials and creating a residue of complex refractory materials that will not sublimate so easily. Study of these mechanisms acting on ice-mantled interstellar dust grains has helped to explain the organic compounds seen in molecular cores⁶ and in cometary comas⁷.

Moreover, two other mechanisms work to modify and/or remove the frosting on the cometary nuclei. Impacts of interstellar dust grains result in micro-

cratering events that appear to remove material faster than it can accumulate^{8,9}, thus resulting in a net erosion of cometary surfaces.

Also, heating of comets by nearby supernovae and by highly luminous young stars that occasionally pass through the Oort cloud has warmed the surfaces of all nuclei to temperatures in excess of the condensation temperature for solid hydrogen and many other volatiles¹⁰. The conclusion is that any solid hydrogen frosting on the nucleus cannot be expected to survive.

The anomalous brightness of new comets might still be explained by volatiles and free radicals created by cosmic-ray irradiation in the near-surface layers of the nuclei which are liberated as the comets warm for the first time on their approach to the Sun. Or it may be an outburst phenomenon resulting from the exogenic conversion of amorphous water ice formed at temperatures below 100 K to crystalline ice, which probably first occurs at about 5 AU inbound¹¹.

Daedalus also errs in saying that the solid hydrogen explosions explain the apparently endless supply of new comets and their random orbits. It is unlikely that the hydrogen explosions could provide sufficient impulse significantly to change the cometary orbits. In reality, the endless supply of new comets is the result of the fact that there are so many of them in the cometary reservoir, of the order of 10^{13} or more¹². Our Sun will have left the main sequence and evolved to a white dwarf star long before the supply is exhausted.

I would hope that this is only a momentary lapse on Daedalus's part, and that his many other interesting proposals in other areas of science with which I am not so familiar are indeed as practical and as near to fruition as he suggests.

Paul R. Weissman

*Jet Propulsion Laboratory,
California Institute of Technology,
4800 Oak Grove Drive,
Pasadena,
California 91109, USA*

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Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

Nature's wanderings

F. Gonzalez-Crussi

Biological Anomalies: Humans I. A Catalog of Biological Anomalies. Compiled by William R. Corliss. *The Sourcebook Project*, PO Box 107, Glen Arm, MD 21057, USA: 1992. Pp. 304. \$19.95.

At the very least, the author of this book will provoke genuine admiration for his disarming optimism. His professed aim is no less than to catalogue the anomalous in the Universe: "all phenomena that cannot be readily explained by prevailing scientific theories". Given the immensity of the road lying ahead, philosophical rigour might have been deemed, one suspects, an encumbrance. Indeed, the definition of anomalous is disposed of in this one-liner, and the determining criteria of what is or is not explainable by current scientific principles are reduced to a pithy sample of unfazed prose: "what in *my* opinion is not well explained. The underlining of 'my' is important, because anomalousness is often in the eye of the beholder." Which it is.

But there are many who are already persuaded that what science 'explains' is a mere patch of light in a boundless expanse of darkness. To this segment of the readership it will come as no surprise to learn that Corliss estimates that his catalogue will require a minimum of 30 thick volumes — he has already published 28 volumes, or 10,000 pages of surveys on "scientific anomalies" — and

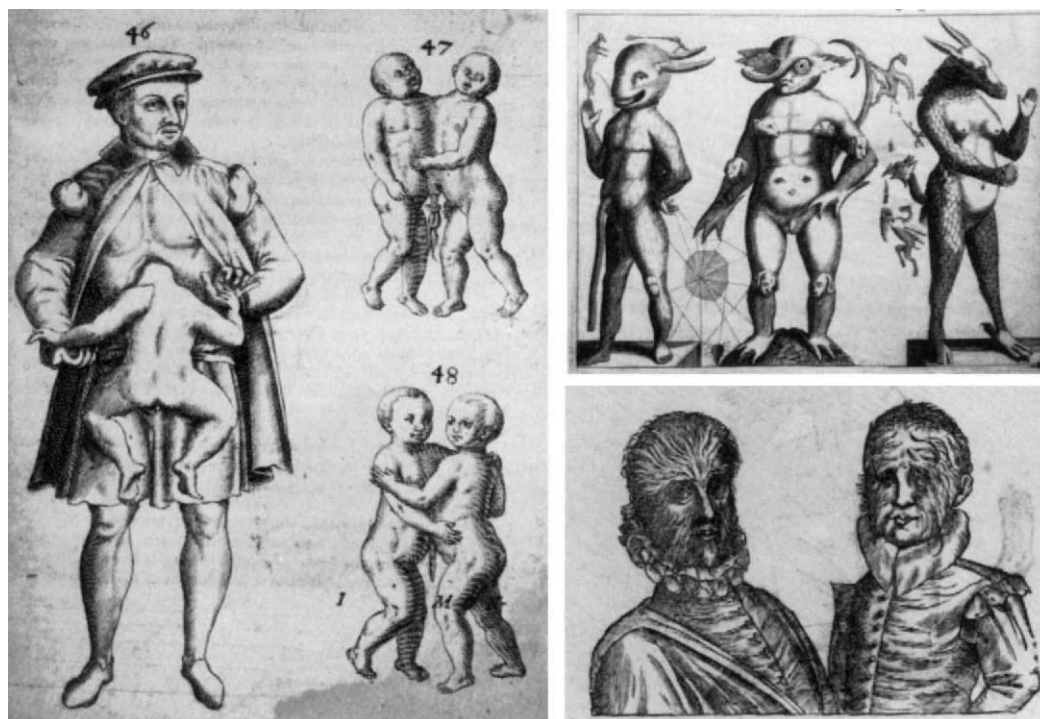
that this represents only about 40 per cent of a database constantly accruing new entries at the rate of 1,200 per year.

Corliss points out that most of his references come straight out of respected scientific journals, such as *Nature*, *Science* and *Human Biology*. A concern to distinguish the viable from the obsolete, however, is nowhere in sight. Moreover, he states that "reports from scientific journals are preferred, but all is grist for the anomaly mill!" None of this, of course, detracts from his laudable intent or astonishing energy. For Corliss, it seems, belongs in the venerable tradition of compilers of 'marvels', with an eye for the prodigious and a unique partiality for the anomalous. Think of Aldrovandi, in sixteenth-century Bologna, bequeathing to posterity a *Monstrorum Historia* in which faithful observations stand side by side with wildly imaginative renderings of mythical beings. Or Liceti's *De Monstris*, where one finds the first report of congenital deformities due to adherences between fetus and amnion, together with fanciful descriptions of beings that existed only in the minds of their creators. Similarly, Corliss juxtaposes well verified clinical observations and titbits from

the popular press.

Like his predecessors, he thinks nothing of confronting single-handedly the anomalous in astronomy, biology, geophysics, mathematics and so on. The latest volume limits its scope to human anomalies, but the author assures us that he is not interested in teratology. In his catalogue, "monstrosities are regarded as biological errors rather than biological anomalies, because no biological laws are challenged." Inevitably, we are reminded of compilers of yore. Georg Pictorius von Villigen (c.1500–1569), physician at the archducal court of Ensisheim in Alsace, wondered why eunuchs have weak sight; why the glance of a menstruating woman spots a clean mirror; why the child born in the eighth month rarely lives; or why there was greater longevity before the Flood. Corliss, the modern compiler, ponders over other puzzles: why the human body is relatively hairless when other mammals have furs; why certain health problems afflict left-handers more often than right-handers; why some humans are born tailed; or whether planetary positions and birth rate correlate with the achievement of superior abilities in some areas of human endeavour.

My prejudice is to expect 'books of wonders' to have a handsome format, adorned by the quaint engravings that decorate ancient bestiaries or the flights of fancy of Albertus Magnus. In this respect, this book is a disappointment. The presentation is homespun, camera-ready, perhaps deliberately wishing to convey the sense of urgency of 'brief communications' that, scientists insist,



SEVENTEENTH-CENTURY books on human 'monsters' tended to be written by physicians, whose interest was mainly teratological. Aldrovandi compiled an exhaustive encyclopaedia of monsters (*Monstrorum Historia*), but its publication (1642) was preceded by the compendia of Schenck (1609) and Liceti (1616). Depicted here: a case of 'parasitic ectopy' (46) and Siamese twins (47, 48) described by Schenck (far left plate); human-animal beasts of Liceti (upper plate); and a woodcut, from Aldrovandi's *Historia*, of two of the Gonzalez family, once well known in Europe for displaying hirsutism (lower plate). These illustrations are taken from more than 250 reproduced in *The Age of the Marvelous* (ed. J. Kenseth), an exhibition catalogue with nine essays exploring European interest in the unexpected, exotic, extraordinary and rare. University of Chicago Press, \$40, £31.95 (hbk), \$29.95, £23.95 (pbk).

brook no delay. Anomalies are first catalogued with a system of letters as belonging to external appearance and morphology (BHA), anomalous human behaviour (BHB) or unusual senses and faculties (BHT). A number is added to identify uniquely the anomaly, making indexing and cross-referencing easy. Thus, "human eyes shining at night" is BHA37, and "eminence in sports champions correlated with the position of Mars" is BHB29. Each item starts with a "description" or definition. This is followed by "data evaluation" in which a numerical score, from one to four, is assigned according to the degree of verisimilitude of the data supporting the observation. "Anomaly evaluation" comes next, a numerical score reflecting the degree to which the phenomenon is a challenge to the established body of scientific knowledge. In vain do we look for reasons to accept these very important scores as trustworthy: we have already been told that the degree of anomalousness is a matter of personal opinion. Additional subsections are "entries", or summaries of illustrative examples culled from the literature, and references.

All these trappings (computer-assisted format, codes of letters and numbers, numerical scores and protestations of giving preference to authoritative scientific sources) might express the author's desire to compel the serious attention of the scientific community. But it is clear that this work must be judged on different grounds. Its cavalier affirmation of subjectivity; its judgemental values; the off-hand use of sources old and recent, current and obsolete, scientific and nonscientific — all these remove the book from the mainstream of science, and place it disturbingly close to folklore and mythology. And this unique emplacement constitutes, to my eye, its greatest strength.

The extraordinary has always evoked in humans the strong emotional responses of wonder, surprise, astonishment and admiration, as well as a profound sense of anxiety: the deviant, the anomalous is sought after, sometimes exalted, often feared. Men deeply desire some form of soothing connection with it, and in this quest are inexorably driven to create myths. Scientists surely have some obligation to know the myths of their own age — if for no other reason than to combat them. And they must expect new ones; for the mythopoeic function is indestructible and its ancient vitality is always ready to sprout new roots. Scientists, if unreflective, tend to ignore or disdain myths, forgetting that myths and dogma can all too easily be built around the very premises of rationalism. That books of this kind continue to appear serves as a reminder

that, in our ever-changing world, rational proof is no guarantee of permanent validity. It is no small paradox that all our rationalist premises demand constant questioning and retrial, and this in order to validate their claims of truthfulness.

There is also a less ponderous reason why this book cannot be dismissed as 'useless' or 'a waste of time'. It is related to the benefit of human warmth, resonance and colour that is annexed to information — including 'wrong' information — which does not directly increase our technical or professional competence. Bertrand Russell claimed that apricots tasted better to him ever since he learned the etymology of the

word 'apricot' and discovered the rich folklore that surrounds this fruit. It is not unwarranted to expect that some researchers will derive greater pleasure from their toils after they find out that handedness has been investigated *in utero* by counting the number of times the fetus sucks the right and the left thumb; that electromagnetic waves have been perceived as sound; or that there is a tendency for the very best athletes to be born as Mars rises in the sky. □

F. Gonzalez-Crussi is in the Department of Pathology, Box 17, Children's Memorial Hospital, 2300 Children's Plaza, Chicago, Illinois 60614, USA.

Techno-dreams (and nightmares)

Daniel S. Greenberg

Route 128: Lessons from Boston's High-Tech Community. By Susan Rosegrant and David Lampe. *Basic Books: 1992. Pp. 240. \$25.*

KEN Olsen, co-founder and still chief of Digital Equipment Corporation (DEC), epitomizes America's postwar alchemy of technological brainpower transmuted into colossal wealth. A graduate of the Massachusetts Institute of Technology (MIT), he was working in the mid-1950s on a defence-funded computer project at the institute's Lincoln Laboratory when he got the itch to go out on his own. With \$70,000 provided by a venture capital company, American Research and Development (AR&D), Olsen and colleagues trod a story-book route: they went into business in rented quarters above a furniture store in Maynard, Massachusetts.

By the mid-1960s, DEC had gone public and AR&D's investment was worth over \$100 million. In 1972, when AR&D was bought out, its DEC shares were valued at more than \$400 million. Olsen, of course, became a millionaire, as well as a legendary figure in the so-called Massachusetts Miracle, a wondrous postwar flowering of high-tech enterprise rooted in the state's academic strength, military laboratories and commercial traditions. Much of the activity generated by that triad was sited on or near a 65-mile stretch of Route 128 encircling Boston and Cambridge. Home to thousands of start-up companies, spin-offs from existing ones, and outposts of distant companies, 128 became the shorthand term for post-industrial enterprise closely linked to university science and technology, entrepreneurship and government contracts, mainly for cold-war technology.

Alas, DEC and many of its electronic

neighbours on that vaunted roadway eventually fell on hard times, and the 'miracle' had faded away by 1989, plunging Massachusetts from the peaks of desirable economic indicators to the lows. The casualties included the presidential ambitions of Governor Michael Dukakis, who, in more prosperous times, had depicted himself as the impresario of the state's soaring good fortune. The forces that produced Massachusetts' high-tech rollercoaster ride are the subject of this book, by two former *Business Week* reporters, one of whom, David Lampe, is assistant director of MIT's Industrial Liaison Program.

The authors list numerous institutional and personal elements situated around '128'. Unfortunately, their analysis of this industrial casserole rarely goes beyond short descriptions of the ingredients. And in places, the text reads like a promotional brochure from the local chamber of commerce, as in: "The story of eastern Massachusetts's high-tech community is a story of idealism and entrepreneurship, of ivory tower intellectualism and practical Yankee ingenuity, of cultures and nations in conflict, and of individual dreams and cooperative efforts. It is a story of the search for new knowledge and the drive to put it to work." They might have added that it is also the story of a particularly influential congressional delegation, consisting of the Kennedys and Tip O'Neill, among others, that diligently routed good things to Massachusetts.

Nonetheless, a basic outline of 128 and Massachusetts' rip-roaring adventure in high-tech prosperity is here. And it is accompanied by appropriate emphasis on the 150 years of preparation — academic, financial and cultural — that enabled the state to respond to the exotic industrial opportunities of the postwar era. Noting that "Massachusetts owes much of its good fortune to microwaves", the authors point out that MIT's

PERFUMERY is considered by many to be an art, presumably because to label it a science is to reduce its powers of enchantment and mystery to an analysis of aldehydes and terpenes, not to mention indoles and salicylates. A science it undoubtedly is, however, with precise factors such as phase partition and critical dilution determining the success of an ephemeral fragrance.

The Perfume Handbook by Nigel Groom skilfully blends whimsy with chemistry, with a fair sprinkling of social and scientific history. Its encyclopaedic format of alphabetic listings (for example, "Low note . . . the third and last phase in the process of a perfume's evaporation on the skin, when the most lasting ingredients, such as woody or animalic scents, become most discernible") disguises a stimulating interpretation of matters olfactory. This is a catalogue, heavily laced with anecdote, of how human responses can be manipulated. Formulas are given for making Arabian incense and the favourite perfume of Henry VIII, and in an appendix some 1,300 modern scents are listed using almost as many adjectives. The book is illustrated with photographs and sketches (shown here are mace and nutmeg, *Myristica fragrans*). Chapman and Hall, £35, \$29.95.

R.C.



pross before the Second World War in electronics research assured a great wartime build-up in that field. In turn, the strength gained in wartime provided a powerful base for postwar industrial developments and the microcomputer boom that lay at the heart of 128's prosperity. Helping too in those early days, they add, was the presence in Washington of Vannevar Bush, a former MIT professor of electrical engineering, as head of the wartime research effort. "With his many MIT connections", the authors state, "it was perhaps inevitable that Bush would think first of his former employer when delegating contracts."

They credit MIT's longstanding cultivation of close ties with industry for providing a natural thrust for faculty and associates to take the plunge into garage-based high-tech enterprise. They note the abundance of Pentagon money in Massachusetts — some \$8 billion in prime contracts, including \$455 million to MIT and associated laboratories in 1990. Many of the small start-up companies in the initial round of 128's growth were launched without the help of venture capital, but private investment later became a considerable factor in the economics of high-tech enterprise in Massachusetts, totalling \$13.4 billion between 1978 and 1984.

Except for several sparse references to Silicon Valley and a few other regions that, in some part, underwent the 128 phenomenon of rapid high-tech growth, the authors focus exclusively on the Massachusetts experience. In doing so, they cover their stated territory, but neglect the opportunity to enlarge our understanding of post-industrial economic growth. Academic strength, entrepreneurship, heavy spending on defence

research and development, and financial acumen are not confined to Boston.

As US presidential candidates joust over the proper role of government in assuring industrial prosperity, it would be helpful to receive useful lessons from the 128 experience. But, although billed in the subtitle of this volume, the offerings are fairly slim, and several of them bear a whiff of regional 'boosterism'. Policymakers are advised that because of the riches of educational and research facilities in the vicinity of Route 128, Silicon Valley and southern California (the last two just mentioned barely), these regions "can provide particularly valuable places for both industry and government to locate advanced technology research facilities, which in turn further encourage the passage of new technologies into the marketplace". And there is the customary pitch for string-free federal money for research, justified on the grounds that "it is dangerous for the federal government to try to out-guess the marketplace or the course of research. It makes more sense to rely on the creativity and entrepreneurship inherent in the system to adapt to meet these needs and demands."

Given the glut of federal money and military goals that underpinned the Boston-area high-tech economy — before the crash, that is — the case for *laissez-faire* is questionable. Rosegrant and Lampe do a good job of telling what happened on that remarkable road that rings Boston and Cambridge. The dynamics of the process are another story. □

Daniel S. Greenberg is editor and publisher of *Science & Government Report*, 3736 Kanawha Street NW, Washington DC 20015, USA.

Brazen anatomy

Fred S. Rosen

Encyclopedia of Immunology. 3 Volumes. Edited by Ivan M. Roitt and Peter J. Delves. Academic: 1992. Pp. 1,578. \$450, £300.

ENCYCLOPAEDISTS are members of an antique profession. Compulsion or hubris or both have long induced scholars to attempt to write down all that is known, *tam sacrarum quam profanum epistemon*, as an encyclopaedist of Basel wrote in 1559. Somewhat later Coleridge noted that an encyclopaedia should contain pure science, applied science, biographical and historical matter, a lexicography and an index. The editors of the *Encyclopedia of Immunology* have assembled both the sacred and the profane from more than 1,200 outstanding scholars and researchers in the field. The contributions, which are 250 to 2,500 words long, maintain a uniform level of conciseness, clarity and excellence. The entries, in a remarkable way, cover the current state of knowledge about immunology. Certainly these three volumes belong on the bookshelf of anyone working in the field.

The editors could have profited from the advice of Coleridge because biographical material would have enlivened this ambitious work. At least, the achievements of the dead and the ennobled could have been described with a succinctness that characterizes the rest of the work and have made this great body of knowledge more vivid.

The volumes might possibly have been even more useful had the entries been arranged by subject matter. The mediaeval Arabic encyclopaedist, Ibn Qutayba, began with power and ended with women. And again Coleridge in his wisdom stated: "To call a huge unconnected miscellany of the *omne scibile*, in an arrangement determined by the accident of initial letter, an encyclopaedia, is the impudent ignorance of your Presbyterian bookmakers." The Calvinist press of the Reformation may not be alone in a state of impudent ignorance.

Immunology began as a branch of microbiology. Its infancy was spent in the description of immunological responses to infection. In this encyclopaedia there is a complete coverage of the immune response to all important infectious pathogens. Nowhere else, to the best of my knowledge, can this information be so quickly and easily obtained. The illustrations, such as an electron micrograph of cryptosporidia resident in the gut of a lamb (or it could be a patient with AIDS), are excellent and indeed worth the proverbial

thousand words.

From its roots in microbiology, immunology became a highly metaphysical exercise 40 years into its life, as scientists began to question the cellular and molecular basis of the immune response to the thousands, if not millions, of antigens that might be encountered by the mammalian host. These metaphysical problems ushered in the golden age of immunology. In a mere 30 years, the cellular and structural basis of the immune response was clarified and the genetic mechanisms that made the diversity of the system possible were deciphered. That done, the central questions posed by immunologists have been answered. Rapid ossification of immunology might have been anticipated at the conclusion of this remarkable progress.

But this has not happened. Important issues of signal transduction and cellular activation have now been put forth and a huge family of adhesion molecules has come to light as cellular interactions in the immune system have been scrutinized. Immunology remains a very lively subject. Traffic from the laboratory bench to the bedside intensifies. It was perhaps brazen to have put this encyclopaedia between hard covers when a loose-leaf format, in which updated pages could replace outworn knowledge or add new information, might have been more viable. The editors assure us in the preface that this is not so. But immunology is not anatomy and only time will convert us to their conviction. □

Fred S. Rosen is at the Center for Blood Research, Inc., 800 Huntington Avenue, Boston, Massachusetts 02115, USA.

Laboratory manuals

- *Immunoassay Automation: A Practical Study Guide* edited by D. W. Chan. Academic, \$49.50, £30.50 (spiral bound).
- *Techniques for the Analysis of Complex Genomes* edited by R. Anand. Academic, \$39.95, £19.50 (spiral bound).
- *Nonisotopic DNA Probe Techniques* edited by L. J. Kricka. Academic, \$39.95, £26.50 (spiral bound).
- *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression* edited by S. R. Gallagher. Academic, \$29.95, £19.50 (spiral bound).
- *Baculovirus Expression Vectors: A Laboratory Manual* edited by D. R. O'Reilly, L. K. Miller and V. A. Luckow. W. H. Freeman, \$49.95 (spiral bound).
- *Gene Expression in Neural Tissues (Volume 9 of Methods in Neurosciences)* edited by P. M. Conn. Academic, \$49.95, £31 (spiral bound), \$95, £59 (hard covers).
- *Virology: A Laboratory Manual* by F. G. Burleson, T. M. Chambers and D. L. Weidbrauk. Academic, \$29.95, £18.95 (spiral bound).

Madness to the method

John W. Galloway

Scientific Literacy and the Myth of the Scientific Method. By Henry H. Bauer. University of Illinois Press: 1992. Pp. 192. \$24.95.

HENRY Bauer has written a book to challenge current ideas about scientific literacy and demolish the myth of the scientific method, a concept that he claims was discarded decades ago by most historians and philosophers of science. Indeed, Peter Medawar pointed out the dangers of belief in the scientific method. For it is the professional business of scientists to solve problems; so those who claim to believe in the method but fail to solve a given problem must either not know the correct method, in which case they are incompetent, or simply be too idle to apply it. Bauer argues that strict adherence to the model of the scientific method leads not only to a stifling of creativity and to bad science, including deliberate fraud, but also to a misunderstanding of science in society.

Bauer begins by looking at how science or, in his words, its reputation works in our culture, exposing some popular fallacies about scientific literacy among the public. Promotion of scientific literacy is beginning to take on the appearance of a major industry, yet he believes that "things are drastically wrong with almost everything that has been said about the supposedly critical state of scientific literacy". He rubbishes the nonsensical rhetoric on the utilitarian value of such literacy, adding that it is no more than part of a public-relations exercise by scientists. There are perfectly good reasons for being scientifically literate, but they are not much different from the benefits of any sort of literacy. It is worth recalling that on learning that the Earth went around the Sun, Sherlock Holmes said that he couldn't see what possible use the knowledge could be to him and now that he knew it, he proposed to forget it. When individuals or organizations really need to know some science, they can become experts in it very quickly. And it is by no means certain that science would benefit if the public understood science better.

But Bauer has more specific concerns. What exactly is meant by scientific literacy? It might mean, as he points out, 'knowing' some science. But how much, and which bits? He notes that different sciences do not often resemble one another a great deal, and not just because of differences in subject matter.

One has only to think of the contrasting ways mathematics is used in theoretical physics and molecular biology (physics relies a great deal for its explanatory power on metaphor).

What's more, as Bauer points out, questions about science cannot easily be reduced to simplistic true-or-false or multiple-choice answers. Here he makes a useful distinction between the science we learn as fact ("textbook science") and the science that is new ("frontier science"), where research is continually creating more knowledge, much of it unreliable and subject to change, and where all fraud takes place. As he says, this division can cause problems for those who rely on the media for knowledge of their science: some people are disturbed to discover that science is just as evanescent as the rest of the news, particularly if they see science as being always dependable.

Certainly, as Bauer contends, it might be more useful for lay people to understand how science works rather than its results. For example, the mistaken but stereotypical view of science as a dispassionate, objective, systematic pursuit of knowledge means that science is perceived as self-regulatory, when in reality, Bauer argues, professional codes of ethics need to be established to deal with, say, cases of transgression. He emphasizes that science is a cooperative, continuously changing and complex enterprise carried out by fallible humans, a social activity or "game" that is an essential part of our cultural heritage; and that it will be of greater benefit to us and better understood when recognized as such.

Bauer develops his theme well, using the myth of method to illuminate many aspects of popular misconceptions about science. But he is better at saying what science is not, defining it by exclusion, than giving a clear idea about what makes it distinctive. Perhaps it is a mistake to look for any general definition of science. In this respect, his adoption of the old analogy between science and games is particularly apt — many games share features, but it is difficult to see what solitaire, chess and football have in common. So, too, with the sciences.

Science can be recognized, however, by the sort of questions it asks and the sort of methods it has to answer them. In the words of Neils Bohr, "it is the task of science to reduce deep truths to trivialities". A large part of scientific creativity is devising methods to do just that. It is by its methods, not its method, that science is known and recognized. □

John W. Galloway is at the Nuffield Foundation, 28 Bedford Square, London WC1B 3EG, UK.

Clues to early Solar System history from chromium isotopes in carbonaceous chondrites

Monica Rotaru, Jean Louis Birck & Claude J. Allègre

Laboratoire de Géochimie et Cosmochimie IPG 4, Place Jussieu 75252, Paris Cedex 05, France

The major mineral phases contained in individual carbonaceous chondrite meteorites exhibit both positive and negative anomalies in their ^{54}Cr abundances. No significant chromium-bearing phase has a terrestrial (solar) chromium isotopic composition. These observations provide evidence that the chromium isotopic composition of the Solar System results from the mixing of several major components with distinct isotopic compositions. The formation of the solar nebula from material carrying different isotopic signatures may provide constraints on nucleosynthetic models.

CARBONACEOUS chondrites are commonly considered to consist of primitive Solar System material, and astronomical observations suggest that the composition of the CI1 chondrites may be typical of the Universe as a whole, not just the Solar System^{1,2}. These 'cosmic' abundances have been taken as strong constraints for nucleosynthetic models³⁻⁷. Nevertheless, deviations from terrestrial (solar) isotopic compositions in carbonaceous chondrites have revealed heterogeneities in the solar nebula⁸⁻¹⁰. Among these anomalies, those for the iron-group elements (this term is used for the elements from Ca to Zn, which are those with the most stable nuclei) are the largest for the neutron-rich isotopes (^{48}Ca , ^{50}Ti , ^{54}Cr , ^{58}Fe and ^{64}Ni)¹⁰. The most anomalous abundances have been reported in refractory material, such as the rare FUN¹⁰⁻¹³ and PSI¹⁴ inclusions of the CV3 Allende and hibonites of the CM2 chondrites¹⁵⁻¹⁸. The anomalies for iron-peak isotopes result from nucleosynthetic processes in the interiors of massive stars^{7,10}. Observed anomalies for neutron-rich isotopes of the iron group in refractory material are roughly correlated^{10-14,16,17,19-21}, because they are synthesized in a neutron-rich nuclear statistical equilibrium^{3-7,22}.

The isotopic variations can be interpreted in two ways. In one view, they result from occasional stellar injections of isotopically unusual material superimposed on a uniform solar (and cosmic) medium. These exotic contributions could be a signature of old events^{7,16,17,22-25} or of a recent input^{8,18,26-29}. The alternative interpretation is that isotope anomalies are common fossils of distinct nucleosynthetic components and that the primitive solar nebula was less homogeneous at the scale of the grain size than commonly thought^{18,30,31}. For the iron group, this model has been proposed as an explanation of the isotopic Ti effects observed in hibonites¹⁸.

But the high-temperature mineral fraction in which these anomalies have been seen makes up only a minor portion of the bulk carbonaceous chondrites. The whole rocks have a near-terrestrial isotopic composition, and despite indications that anomalous ^{50}Ti is not confined to refractory assemblages^{25,32}, the ratios of iron-group isotopes in the low-temperature material that constitutes the matrices have not been extensively investigated.

Here we report ^{54}Cr data obtained for bulk carbonaceous chondrites dissolved in acids whose strength was increased in steps. Even if this procedure does not precisely determine the compositions of the dissolved minerals, it allows us to identify the chromium isotopic components in the major mineral phases, which exists as submicrometre grains in the matrix. We find that both positive and negative ^{54}Cr anomalies occur in every sample of CI1 or CM2 chondrite. The internal heterogeneity decreases with increasing petrographic grade, reflecting the effect of secondary metamorphism in parent bodies. But in Orgueil (CI1), no significant Cr-carrier has terrestrial (solar) isotopic composition.

These observations strongly support the idea that the solar nebula was highly heterogeneous. As Cr is present in solar proportions overall in carbonaceous chondrites, our data obtained from major mineral types give the first evidence that the isotopic composition of material representative of the Solar System is a mixture.

In contrast to what would be expected from refractory inclusions¹⁰⁻²¹, our procedure does not reveal any isotope anomaly for the other iron-peak elements. This implies that detection of isotopic heterogeneities depends on the way that freshly synthesized isotopic compositions are incorporated into minerals. We argue that the pattern of isotope anomalies in meteorites implies the existence of multiple nucleosynthetic sites, and different condensation processes associated with the different sites.

Selective dissolution of minerals

We analysed nine carbonaceous chondrites, ranging in metamorphic grade from C1 to C4. Their main features are listed in Table 1. The matrix extracts are probably not pure in each case, but the enrichment in matrix material during the separation, together with the minor content of Cr in refractory inclusions¹⁹, ensures that the high-temperature components remaining in our samples do not significantly affect the apparent composition of the matrix.

The isolation of mineral phases from bulk rocks has received special attention from workers investigating carriers of anomalous rare gases (see ref. 33 for a review). Our purpose was to separate and analyse the main Cr-bearing phases included in a sample. We used a stepwise dissolution procedure because major mineral matrix phases are submicrometre in size and therefore difficult to extract by traditional mineral separation procedures. The mineralogical nature of the dissolved phases in each leachate was estimated from known mineral and acid properties. Major element concentrations (not measured) would provide a more precise characterization of the minerals dissolved in each step but are not essential for the discussion of Cr isotopes.

The powdered sample was subjected to a sequence of dissolution steps with reagents of increasing strength. This procedure yielded solutions numbered from 1 to 5. The two first steps dissolved easily soluble phases (carbonates, sulphates, sulphides and so on), and the later fractions contained more acid-resistant phases such as hydrated or anhydrous silicates (step 4) and spinels (step 5). The residue (<2 wt%), which was similar to that obtained elsewhere at a corresponding degree of attack³³, contained carbon components³³. Analytical procedures^{19,34,35} are described in Table 2.

The procedure was subdivided into further steps (steps 2', 2'' and so on), in particular for Orgueil-III, to obtain better separation of the isotopic components.

Results

The chromium contents establish the relative importance of the isotopic components in the different dissolved phases. Chromium (Table 2) is mainly concentrated in the less acid-resistant fractions for the CI1 chondrites (>80%), and in the most acid-resistant fractions for the C3 and C4 chondrites (94%). Its distribution in the CM2 chondrites is more variable. In contrast with Cr, Mn is concentrated in the less acid-resistant phases (80%) for all samples. This Mn is probably derived from carbonates, sulphates and sulphides dissolved in steps 1 to 3.

Dissolved fractions display positive and negative deviations in ^{54}Cr relative to terrestrial (solar) composition. The data are quoted in ϵ -units (relative deviation in parts per 10^4). These opposite isotope anomalies are found in different mineral types from the same meteorite (Table 2; Fig. 1). The reproducibility for the leachates is satisfactory. Positive anomalies in ^{54}Cr exist in C1 to C4 chondrites. The excess is as large as $+100\epsilon$ in Orgueil (CI1); it decreases to 27ϵ for Murchison or Murray (CM2), and to 2.8ϵ in Felix (CO3) and Coolidge (CV4). (The

TABLE 1 Experimental procedure

Reagent	Step number	Procedure	Comments	Dissolved phases
Acetic acid (17N)	1	2.5% 30 min 20 °C		Sulphates, carbonates, sulphides, metal, ...
	2	50% 24 h 20 °C	Orgueil-III 2': 20%; 2 h 2'': 50%; 12 h	
Nitric acid (16N)	3	25% 7 days 20 °C	Orgueil-III 3': 3%; 10 min 3'': 6%; 1 h 3''': 25%; 12 h	Mixture
	4	1/1 4 days 100 °C	Orgueil-III 4': 25%; 2 h; 20 °C 4'': 4 days; 100 °C Coolidge, B-7904 4: 4 days; 20 °C Karoonda-III 4: 30 days; 20 °C	Silicates
HNO ₃ (16N) + HF (27N) or HBr (9N) + HF (27N)	5	HBr/HF: 95/5 10 days 150 °C	Coolidge, B-7904 HNO ₃ /HF: 1/1; 4 days; 150 °C	Refractory phases such as spinels

CI1 chondrites consist mainly of low-temperature matrix, crossed by secondary veins (carbonate, sulphate) produced by aqueous alteration. The matrix (99 vol%) contains principally magnetite (11 wt%), phyllosilicates and a variable amount of ferrihydrite⁴⁷. We selected Orgueil (four samples), Ivuna and Alais which has suffered from terrestrial weathering. The CM2 chondrites contain more high-temperature material (mean 40%), as chondrules, individual minerals and refractory inclusions. Phyllosilicates are again a major constituent of the matrix. In sampling, care was taken to avoid large inclusions and chondrules. We investigated Murchison (four replicates), Murray and Belgica 7904. The classification of the Antarctic meteorite Belgica 7904 (B-7904) is not well defined: petrologically it seems to be a CM2 chondrite, but its oxygen isotopic composition⁴⁸ is close to the range of the CI1 chondrites. The matrix of the C3 chondrites, less abundant than in CM2 chondrites, is essentially made of fine-grained Fe-rich olivine. A matrix sample was separated for Allende (CV3). We made no attempt to extract the numerous small high-temperature objects in Felix (CO3) and instead analysed the bulk rock. Few carbonaceous specimens have a petrological type greater than 3. We analysed Coolidge (CV4) and three replicates for Karoonda (CK4)⁴⁹. The samples represent bulk rocks, because the distinction between matrix and small inclusions becomes unclear owing to recrystallization under metamorphism. Between leaching steps, the powdered sample was carefully rinsed with distilled water to avoid overlapping of the different isotopic components. Dilute acetic acid dissolves easily soluble phases. Dilute nitric acid for step 3 completes their dissolution. Steps 4 and 5 correspond to the usual procedure for dissolving silicates and refractory minerals. The 'Comments' column lists differences in the procedure. Whole rocks are dissolved in boiling HF + HCl and HF + HBr.

excess of 12.6ϵ in Karoonda is associated with a minor proportion (>0.1%) of the total Cr content, resulting in poorer precision). The silicate fraction in the CI1 and the refractory phases in the CM2 chondrites are the most enriched in ^{54}Cr . Negative effects occur in the easily soluble phases of the least-metamorphosed chondrites. The deficits are -6.65ϵ for Orgueil (the previously reported³⁶ value of -16ϵ was never duplicated) and -16ϵ for the CM2 chondrites. The internal isotopic heterogeneity decreases with increasing petrological type. No significant Cr carrier has the terrestrial Cr isotopic composition. This is emphasized by the subdivided procedure applied specifically to Orgueil-III (inset to Fig. 1a). But when all the components are taken together, the bulk values calculated from the data are $1.22 \pm 0.70\epsilon$ for Orgueil, $-0.39 \pm 0.70\epsilon$ for Ivuna, $0.70 \pm 0.70\epsilon$ for Murray, $1.71 \pm 0.70\epsilon$ for Allende. These results are close to the terrestrial value and agree with whole-rock ^{54}Cr compositions.

Anomalies in ^{53}Cr are smaller than in ^{54}Cr . The largest ^{53}Cr enrichments occur in the Mn-rich fractions. There is no correlation between ^{54}Cr and ^{53}Cr anomalies. In particular, excesses in ^{53}Cr are found in mineral types resulting from processes that mobilized the Mn-Cr system in the parent bodies: aqueous alteration for the CI1 chondrites (fraction 1) and metamorphism for the CK4 chondrite Karoonda.

Isotope data on refractory inclusions¹⁰⁻²¹ and nucleosynthetic calculations^{4,22} indicate that ^{54}Cr anomalies should be associated with detectable effects on the other heavy isotopes of the iron peak. We therefore measured the isotopic composition of Ti, Fe, Ni and Zn for the fractions that had the greatest ^{54}Cr anomalies. The techniques will be described elsewhere. The precisions are 5ϵ , 5ϵ , 1ϵ and 0.2ϵ for ^{50}Ti , ^{58}Fe , ^{64}Ni and ^{66}Zn respectively. For fractions 2 and 4 of Orgueil, Murchison and Murray, very small positive anomalies³⁷ are present for ^{66}Zn and normal values are obtained for the other elements within experimental precision³⁸. For Ti, only fraction 4 contained sufficient Ti to be measured within 5ϵ : a normal isotopic composition was found³⁸.

Solar mixture

Our data indicate that the Cr 'solar value' as estimated from the CI1 chondrites is an average from distinct components with ^{54}Cr excesses and ^{54}Cr deficits. Positive and negative effects have been previously reported in refractory material⁸⁻²¹, but this represents only a small part of the Cr budget of the whole rock. Here we demonstrate that the bulk of the primitive carbonaceous chondrites is made of major mineral types that are isotopically anomalous for Cr. The preservation of both excesses and deficits in ^{54}Cr in neighbouring regions of the same sample supports the idea that the early Cr carriers were already in solid form and that dust grains were the main carriers of most of the elements^{10,15,39}.

Because there is no correlation between ^{53}Cr and ^{54}Cr anomalies in our data, we first discuss ^{54}Cr isotopic anomalies in relation to the other neutron-rich isotopes of the iron group, and then consider them in relation to ^{53}Cr .

Nucleosynthesis and anomaly detection

The isotopes of the iron-peak elements are thought to be essentially synthesized within massive stars at the end of their life^{3,7,22}. Nuclear statistical equilibrium calculations for a range of neutron enrichments^{4,22} reproduce isotope anomalies observed in the neutron-rich nuclides of the iron peak in refractory inclusions¹⁰⁻²¹. Within this framework the ^{54}Cr enrichments in refractory inclusions agree with excesses in the other neutron-rich isotopes of the iron peak. If this correlation applied in the present experiments, a ^{54}Cr excess of 100ϵ for the silicate fraction of Orgueil would correspond to detectable excesses for ^{50}Ti and ^{58}Fe of 115ϵ and 30ϵ , respectively. For the ^{54}Cr negative anomalies, as for the FUN-type inclusions of Allende^{11,14}, only the components reflecting lower neutron fluxes are present.

TABLE 2 Isotope and concentration data

C11					CM2					C3 & C4				
	ϵ_{53}	ϵ_{54}	Mn*	Cr*		ϵ_{53}	ϵ_{54}	Mn*	Cr*		ϵ_{53}	ϵ_{54}	Mn*	Cr*
Orgueil-I					Murchison-I					Allende‡				
WR	0.62±0.20	1.16±0.70			1	0.17±0.16	-8.71±0.70			WR	0.14±0.26	1.70±0.70		
1	1.70±0.20	-1.66±0.70			2	0.47±0.25	-15.77±0.70			1	0.69±0.20	1.84±0.70	9	7
2	0.77±0.20	-7.14±0.70			3	-0.34±0.27	4.56±0.70			2	1.80±0.23	-0.74±0.70	1,100	52
3	-0.12±0.25	-6.52±0.70			4	-0.08±0.41	17.30±0.89			3	0.14±0.20	1.30±0.70	190	178
4	-0.22±0.20	94.96±0.70								4	0.67±0.38	1.76±0.78	273	3,733
5	-1.43±0.20	23.13±0.70			Murchison-II					Felix				
Orgueil-II					1	0.04±0.20	-9.10±0.70			1	4.33±0.88	7.47±2.06	47	1
1	2.78±0.23	-2.71±0.70	640	17	2	0.00±0.23	-15.79±0.70			2	2.86±0.26	2.87±0.70	1,181	28
2	0.90±0.20	-5.61±0.70	590	665	3	0.53±0.22	2.02±0.70			3	0.49±0.72	1.45±1.35	518	96
3	0.30±0.20	-4.69±0.70	340	1,247	Murchison-III					4	0.19±0.26	2.05±0.70	395	3,142
4	-0.33±0.36	101.45±0.70	32	61	1	0.99±0.26	-8.10±0.70			Karoonda-I§				
5	-1.18±0.23	53.60±0.70	8	112	2	0.53±0.50	-16.11±0.85			1	0.16±0.50	4.24±1.03		
Orgueil-III					3	-0.13±0.25	3.25±0.70			2	18.20±0.52	9.81±1.13		
1	1.85±0.30	-2.06±0.70			4	-0.20±0.20	17.58±0.70			3	1.97±0.28	0.18±0.70		
2'	1.82±0.20	-2.04±0.70			Murchison-IV					4	-0.14±0.25	0.76±0.70		
2''	0.49±0.20	-4.66±0.70			1	0.47±0.20	-8.37±0.70	140	63	Karoonda-II				
3'	1.07±0.20	-4.22±0.70			2	0.79±0.32	-13.73±0.70	789	776	1	0.22±0.49	0.84±1.10		
3''	0.89±0.20	-7.69±0.70			3	0.04±0.24	4.54±0.70	319	610	2	24.32±3.08	13.58±5.67		
3'''	0.54±0.20	-7.51±0.70			4	0.06±0.20	15.05±0.70	224	502	3	3.91±0.38	2.40±0.74		
4'	1.67±0.26	32.72±0.70			5	-0.50±0.33	27.42±0.70	11	210	4	-0.01±0.43	0.32±0.82		
4''	-0.72±0.29	88.05±0.70			Murchison					Karoonda-III				
5	-0.99±0.30	12.42±0.70			WR†	0.20±1.00	1.10±1.30			1	0.94±0.35	-1.27±0.78	15	3
6	-0.82±0.20	7.72±0.70			1 mean	0.36±0.20	-8.58±0.70			2	11.86±1.08	12.58±2.27	346	2
Orgueil-IV					2 mean	0.36±0.20	-15.49±0.70			3	32.07±0.25	8.40±0.70	759	6
1	2.77±2.72	-5.43±4.36			3 mean	-0.12±0.20	3.40±0.70			4	-0.01±0.23	1.52±0.70	153	2,056
2	0.71±0.20	-7.64±0.70			4 mean	-0.06±0.20	15.95±0.70			Coolidge				
3	0.02±0.20	-6.85±0.70			5 mean	-0.50±0.33	27.42±0.70			1	1.01±0.42	3.30±0.96		
4	-0.62±0.28	92.99±0.70			Murray					2	0.90±0.20	2.83±0.70		
Alais					WR	0.04±0.24	1.69±0.70			3'	0.78±0.28	0.28±0.70		
1	2.48±0.35	-1.99±0.78	468	13	1	0.41±0.20	-7.24±0.70	170	65	3''	0.76±0.24	2.34±0.70		
2	1.22±0.28	-3.72±0.70	622	595	2	0.80±0.20	-17.60±0.70	776	927	4	0.23±0.26	0.89±0.70		
3	0.55±0.24	-3.37±0.70	1,028	1,139	3	-0.24±0.20	9.29±0.70	266	408	5	0.10±0.26	1.95±0.70		
4	-0.78±0.20	56.34±0.70	28	179	4	0.29±0.22	15.44±0.70	290	612					
5	-0.85±0.27	8.12±0.70	29	184	5	-0.25±0.21	27.64±0.70	10	184					
Ivuna					B-7904									
WR	0.15±0.26	-0.07±0.70			1	1.32±0.22	-5.39±0.70							
1	4.13±2.03	-2.83±3.86	790	11	2	0.34±0.20	-10.02±0.70							
2	0.42±0.24	-7.62±0.70	554	725	3'	0.30±0.37	-3.58±0.78							
3	0.06±0.26	-5.55±0.70	372	1,113	3''	0.19±0.29	-2.70±0.70							
4	-0.82±0.20	79.56±0.70	37	111	4	0.21±0.20	3.12±0.70							
5	-0.68±0.29	22.73±0.70	6	92	5	-0.27±0.20	14.03±0.70							

Isotope analyses were done with a solid-source thermal-ionization mass spectrometer. WR stands for whole rock. Instrumental mass fractionation is corrected using the NBS (ref. 50) value $^{52}\text{Cr}/^{50}\text{Cr}=19.2831$. Our current mean standard values (R_{std}) after correction are $^{53}\text{Cr}/^{52}\text{Cr}=0.1134556\pm0.0000005$, $^{54}\text{Cr}/^{52}\text{Cr}=0.0282116\pm0.0000002$. Isotopic data and errors (2 σ) are quoted in ϵ -units: $\epsilon_i=(R_i/R_{\text{std}}-1)\times10,000$ where R_i is the mean $^{i}\text{Cr}/^{52}\text{Cr}$ ratio. The current precisions for ϵ_{53} and ϵ_{54} are 0.2 ϵ and 0.7 ϵ , respectively. Within an experimental resolution of 3‰, there is no evidence of mass fractionation in our data for the main Cr components.

* Mn and Cr concentrations were measured in at least one meteorite of each petrographic grade by graphite furnace atomic absorption spectrophotometry. The validity of the technique for Cr concentration measurements has been checked by isotope dilution. The concentrations are given in p.p.m. and are calculated using the initial sample weight. The uncertainty is estimated to be lower than 5%. The total contents deduced from the leachates may differ from the literature⁵¹ (up to 30% for the CM2s), because of losses of fines during rinsing.

† Bulk isotopic composition for Murchison from ref. 11.

‡ A residue corresponding to step 5 for Allende previously gave a Cr isotopic composition close to that of the whole rock¹⁹.

§ Step 5 was not done for Karoonda.

The lack of correlation among the neutron-rich isotopes of the iron peak in a meteoritic sample containing components with two distinct Cr isotopic ratios leads one to wonder why such isotope heterogeneities are not more widely seen for other elements. A solid assemblage of cosmic dust grains would contain various mineral and chemical compositions (silicates, oxides, metals, carbides and so on)²⁸, as reported from space observations⁴⁰. These grains are of diverse nucleosynthetic origins and may carry distinct isotope signatures^{7,23,24}.

The ability to detect isotope anomalies in a meteoritic sample enclosing such grains depends on the size of the analysed sample (X) relative to the size of the grains (x). Variations can be detected only if the ratio (X/x) is small. As the size of interstellar grains is on average⁴¹ 0.1 μm , a 1 mm³-sized sample contains $\sim 10^{12}$ grains. Even if each grain contained only one Cr isotope, and the grains were randomly mixed, the expected statistical

spread around the average is only $\sim 0.01\epsilon$, which is well below present-day experimental possibilities.

Two cases will improve our chance of detecting distinct isotope signatures. First, interstellar grains with the same isotope signature agglomerate in clumps before mixing with other cosmic dust grains. If the agglomerates are of millimetre size, then random sampling at this scale will permit detection. Such may be the case for Allende inclusions precursors³⁹. Isotope ratios in the refractory inclusions will not be clearly correlated because they have been disturbed as the inclusion materials evolved from the interstellar medium to their accretion within a rock.

Second, different nucleosynthetic phenomena produce different isotope signatures. Each source yields similar elements, but these will condense in different minerals, reflecting the specific physicochemical environment of each nucleosynthetic site (for example, different C/O ratios^{24,28}). A leaching

experiment that dissolves a single specific mineral type will detect anomalies, and if the isotopic distribution is variable, both positive and negative anomalies will be observed. This seems to be the case for Cr here (Fig. 2a).

The situation is different for an element that is produced by different nucleosynthetic sources but ends up in the same mineral type (Fig. 2b). Stepwise dissolution will etch all minerals of this type together, despite their different histories, because of their similar chemical nature, and will therefore produce a mixture of the different isotope signatures. This may be the case for Ti and Fe. This situation is supported for Ti by the theoretical condensation sequence which predicts that this element is trapped in early refractory grains and by the experimental observation that our fraction 4 is the only one that contains enough Ti to allow a precise isotopic measurement. The Fe results are more puzzling. We know that this element is present (to various

degrees) in all our solutions from their typical yellow colour, but it is possible that this reflects a secondary distribution of Fe after aqueous alteration from the initial precursors (in particular, the metal and sulphide). The behaviour of Cr relative to that of Fe during aqueous alteration requires further work. The important conclusion of this section is that cosmic chemistry, and especially the way in which elements are distributed in cosmic minerals, is fundamental to understanding isotope distributions in meteorites: the anomalies depend both on nucleosynthesis and cosmic chemistry^{7,24}.

In addition to the problem of resolution, a second effect that tends to erase initial isotope heterogeneities is thermal diffusion. For low-temperature metamorphism (<400 °C), the diffusion coefficient, D , for Fe and Ni in solid silicates⁴² $\sim 10^{-19}$ cm² s⁻¹. D for Cr is likely to be similar. This temperature allows a mass transport distance, $x = \sqrt{Dt}$, of 1 μ m (10 times the grain size) in only 3,000 yr. It is not surprising, therefore, that metamorphosed meteorites seem rather isotopically uniform. The correlation between decreasing isotope anomalies and higher petrologic type reflects greater re-equilibration under more intense metamorphism. According to this scenario, the resistant minerals of the CM2 meteorites (fraction 5) have retained the signature of their initial isotopic composition better than the phyllosilicates (fraction 4), when compared with the 'original' composition in the CI1 chondrites. From the above estimate of diffusion rate, it turns out that it is easy to rehomogenize the isotope distribution in this material, so we must ask how long such heterogeneities would persist in the interstellar medium and the solar nebula.

Several factors indicate that the initial isotopic anomalies could have been very large: oxygen anomalies for refractory inclusions suggest pure ¹⁶O addition for one endmember⁸; the ⁵⁰Ti anomalies increase with decreasing sample size from a few ϵ in whole rocks^{25,32}, through 10–100 ϵ in small samples^{43,44} to 700–2,700 ϵ with ion probe spots^{15–18}; and given the tendency towards homogenization, the persistence of Cr anomalies suggests that they must have been large initially. Analyses reveal only small isotopic variations because the carriers have been destroyed or mixed with other components. Previous work on Ti and Cr isotopes has shown that at least four distinct nucleosynthetic components are required to explain the isotopic compositions encountered in refractory inclusions^{11,17,25}. The question which must now be addressed is: when were these components made? It is known from the presence of short-lived nuclides in the early Solar System that fresh nucleosynthetic inputs lasted almost until Solar System formation (that is, within a few million years beforehand)^{19,27,29}. The relative proportion of material coming from such late nucleosynthetic events in the solar mixture is still an open question.

Nucleosynthesis and time

There are many arguments in favour of significant recent inputs. In our dataset, the ⁵³Cr anomalies are well correlated with the Mn/Cr ratio, if we take into account the fact that some observed deviations from the correlation are caused by aqueous alteration and by our leaching procedure³⁸. In our opinion, ⁵³Cr anomalies mainly arise from *in situ* ⁵³Mn decay^{19,35,38,45}. This aspect will be discussed elsewhere (M.R., J.L.B. and C.J.A., manuscript in preparation).

In most models of nucleosynthesis, the iron-peak elements are essentially supernova products: ⁵⁴Cr is synthesized in sites with large neutron densities, whereas ⁵³Cr is the radiogenic daughter of ⁵³Mn (half-life 3.7 Myr) which is produced in sites with lower neutron fluxes. There may also be marginal production of ⁵³Mn in sites producing mainly ⁵⁴Cr (ref. 4). This latter possibility is in agreement with the observation that ⁵³Cr anomalies are generally much smaller than ⁵⁴Cr effects, although there are two exceptions: the inclusion¹¹ EK 1-4-1, and the phases of very high Mn/Cr ratio of Karoonda^{38,45}. The connection between the short-lived ⁵³Mn and the stable ⁵⁴Cr would

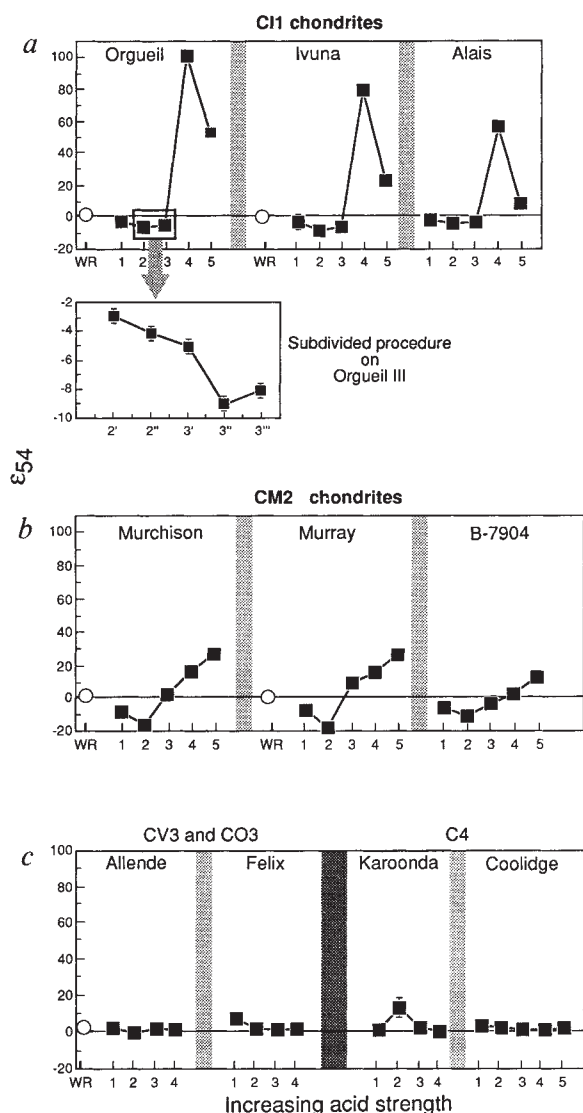


FIG. 1 Anomalies in ⁵⁴Cr as a function of leaching step number. All the data are plotted at the same relative scale. WR represents the whole-rock composition. *a*, Largest ⁵⁴Cr heterogeneities are seen in the CI1 chondrites. The smaller variations in Alais may result from terrestrial weathering. The excess in the silicate fraction of Orgueil is twice that encountered in the FUN inclusion¹¹ EK-141 and may be compared to the largest deficit found in FUN-type inclusions¹⁴. *b*, Intermediate heterogeneities are seen for the CM2 chondrites. B-7904 seems more re-equilibrated than the other CM2 chondrites. *c*, Smallest ⁵⁴Cr heterogeneities are seen for the C3 and C4 chondrites. Felix-1 and Karoonda-2 contain little Cr and were analysed under extreme conditions.

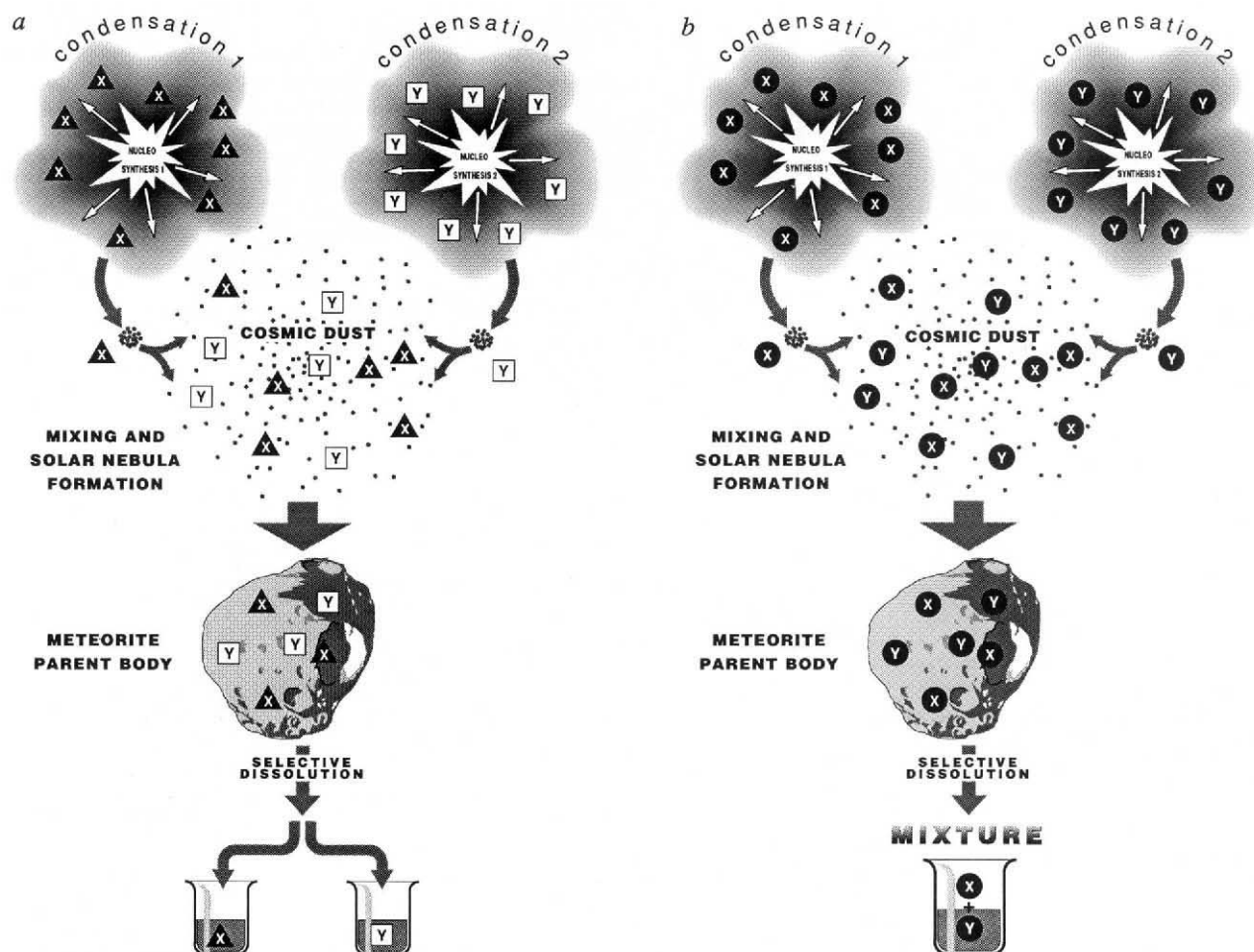


FIG. 2 Possible explanation for lack of correlated isotope anomalies in the matrices. *a*, Chromium. Each nucleosynthetic site produces its specific isotopic composition (X for site 1, Y for site 2) which condenses in one mineral type (a triangle for 1 and a square for 2). The mineral types are later mixed and accrete into a rock. A selective leaching procedure dissolves

the two mineral types separately, so the different isotopic compositions X and Y are resolved in different steps. *b*, Titanium (or iron). The compositions condense in the same mineral type in both sites (represented by a circle). A leaching procedure dissolves all these grains together because of their similar chemical nature. The isotopic compositions X and Y are thus mixed.

establish a temporal relationship between the formation of carbonaceous chondrite and the nucleosynthetic events that produce the anomalies in ^{54}Cr and probably in the other neutron-rich isotopes of the iron group in refractory materials.

We consider the hypothesis that ^{53}Mn is a byproduct of ^{54}Cr as unlikely, although it cannot be discarded. The most probable case is that the ^{53}Mn producing the ^{53}Cr anomalies formed in a low-neutron zone in a massive star which ends as a supernova. In this case we are faced with another question: how much ^{54}Cr is produced in the same event? From the data, the amount is not quantifiable but the conclusion remains that some of the ^{54}Cr anomalies result from the same input as ^{53}Mn .

The half-life of ^{53}Mn is 3.7 Myr, and the isotope has been found to exist in the early Solar System^{19,35}. It is therefore possible that there were large inputs of neutron-rich isotopes³¹ to the solar nebula less than 20 Myr before the formation of the Solar System (^{53}Mn from earlier inputs would have decayed completely). Another argument in favour of inputs of young material, as developed above, is the sensitivity of isotopic heterogeneity to rehomogenization. In addition, the short exposure age of SiC presolar grains may indicate that the Solar System formed in part from atypically young material⁴⁶. Titanium isotope anomalies observed in refractory inclusions or in hibonites have been interpreted as relicts of old events^{16,17,25}; but 'old' in this case means not less than a few

million years. On the other hand, Ti isotope anomalies in hibonites have also been considered as signatures of recent inputs¹⁸.

Together with the presence of ^{53}Mn in material that also shows widespread ^{54}Cr heterogeneities, this set of features raises the possibility that a considerable part of the primitive solar nebula, up to several per cent, was synthesized within a few million years before the formation of the Solar System.

Conclusions

Chromium isotope data show that the bulk solar composition results from the mixing of distinct nucleosynthetic components which are typically supernova products. Many models of the overall production of supernovae try to reproduce the Solar System in each event³⁻⁷. As the solar nebula may have many components and as much of this material may be young, we suggest instead that models should be developed for multiple supernovae each producing a specific signature, solar values being a mixture of extreme endmembers.

The isotope composition data of early materials allows the following sequence of events to be established for the early history of the Solar System. First, isotopes of the iron peak are synthesized in supernovae. At least one of these massive stars exploded a few million years before the formation of the Solar System. Next, condensation produces some grains close to the nucleosynthetic sites. Supernova envelopes are an attractive

location for these processes. Supernovae explosions then inject new populations of solid grains into space, sometimes as aggregates which carry specific isotope signatures. These grains have a variety of chemical compositions. They become mixed with grains of different origin and with different isotope signatures,

to build up the solar isotopic composition. All of these grains accrete to produce meteoritic material, and secondary heating, within meteorite parent bodies, tends to homogenize the isotope record. The most primitive meteorites (C1, C2) keep, at least partially, the memory of their initial isotopic diversity. □

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The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*

Russell J. Hill & Paul W. Sternberg

Howard Hughes Medical Institute and Division of Biology, California Institute of Technology, Pasadena, California 91125, USA

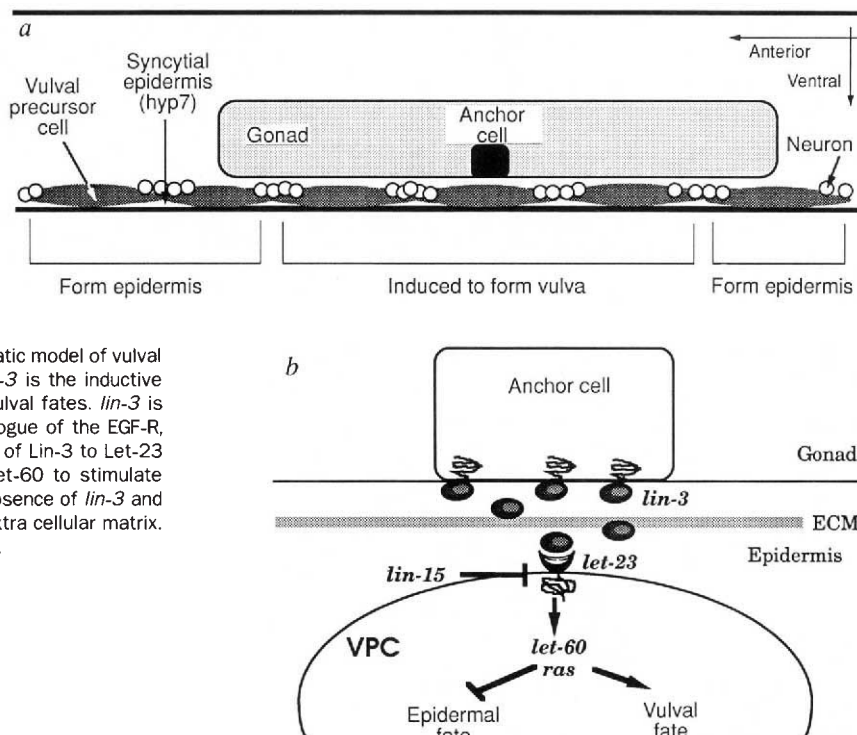
The *lin-3* gene is necessary for induction of the *Caenorhabditis elegans* vulva by the anchor cell. It encodes a molecule similar to epidermal growth factor and to transforming growth factor- α and acts through the epidermal growth factor receptor homologue *let-23*. Expression of *lin-3* in the anchor cell stimulates vulval induction; *lin-3* may encode the vulval inducing signal.

INDUCTION is a general developmental mechanism in which one set of cells specifies the fate of another set of cells. The vulval development of *C. elegans* hermaphrodites is an intensively studied example of induction (reviewed in ref. 1). The vulva normally forms from a subset of six tripotential cells called the vulval precursor cells (VPCs) that are located within the ventral epidermis. Each VPC can have an epidermal fate in which it produces two progeny that fuse with the syncytial epidermis, hyp7. Alternatively, each VPC can have either of two vulval fates in which it produces seven or eight vulval progeny. In a wild-type animal, the three VPCs that are closer to the anchor cell of the somatic gonad have vulval fates and the three VPCs farther from the anchor cell have epidermal fates (Fig. 1a). If the anchor cell is destroyed early in development, then all six VPCs have the epidermal fate, indicating that the vulval fates are induced by a signal from the anchor cell². This vulval inductive signal can act directly on each VPC and may be spatially graded³. This vulval inductive signal may be a secreted

factor because vulval induction can occur in *dig-1* mutant animals when there is no apparent contact between the anchor cell and the VPCs⁴.

Many genes involved in the process of vulval induction have been defined by mutations that fall into two broad classes¹. Vulvaless (Vul) mutations decrease the number of VPCs that assume vulval fates, whereas multivulva (Muv) mutations increase the number of VPCs that assume vulval fates. Mutations that partially lower *lin-3* activity confer a Vul phenotype in which the VPCs that would normally form the vulva instead usually form epidermis, even though the anchor cell is present^{5–8}. Although *lin-3* affects other developmental processes, for example a putative null mutation in *lin-3* results in lethality early in postembryonic development⁶, only the role of *lin-3* in vulval development is characterized here. A genetic pathway of vulval induction has been derived from the epistasis displayed by the Vul and Muv mutations^{7,9,10}. These experiments suggest that *lin-3* acts before *let-23* and *let-60*, which are other genes

FIG. 1 Model of vulval induction. *a*, A schematic diagram of the anatomy of an early L3 *C. elegans* hermaphrodite at the time of vulval induction. Each of the six vulval precursor cells (VPCs) located within the ventral epidermis has the potential to assume an epidermal fate or either of two vulval fates. The anchor cell (AC) is located ventrally in the somatic gonad and is the source of an inductive signal necessary for the VPCs to assume vulval fates. In wild-type animals the three VPCs closest to the AC (P[5-7].p) assume vulval fates and the three other VPCs have epidermal fates. The AC and the VPCs are separated by the body cavity. This drawing is based on anatomical studies described in ref. 39 and work by L. Carta (personal communication). *b*, Schematic model of vulval induction. As described in the text, we propose that *lin-3* is the inductive signal from the AC necessary for the specification of vulval fates. *lin-3* is genetically upstream of *let-23*, which encodes a homologue of the EGF-R, and of *let-60*, which encodes a Ras protein. The binding of Lin-3 to Let-23 is proposed to activate Let-23 which then activates Let-60 to stimulate vulval fates. *lin-15* appears to inactivate *let-23* in the absence of *lin-3* and may originate from the surrounding epidermis³⁸. ECM, extra cellular matrix. Based on the studies reviewed in ref. 1 and our results.



necessary for vulval induction; *let-23* encodes a homologue of the epidermal growth factor receptor (EGF-R) subfamily and is the proposed receptor for the inductive signal^{9,11}, and *let-60* encodes a *ras* protein that acts downstream of *let-23* as a switch to control VPC fate^{10,12,13}. Several other genes that are believed to act downstream of *lin-3* during vulval induction have been molecularly characterized: *lin-10* encodes a novel protein¹⁴ that is necessary for wild-type levels of vulval induction; *sem-5* encodes a novel protein with SH2 and SH3 domains¹⁵. To establish further the role of *lin-3* in vulval development, we have cloned the *lin-3* locus and examined the genetic properties of *lin-3* transgenes. Our analysis indicates that *lin-3* has a structure similar to certain mammalian growth factors, and that *lin-3* can act as a signal *in vivo*.

Cloning of the *lin-3* locus

We cloned the *lin-3* locus by transposon tagging and DNA-mediated transformation (Fig. 2). We obtained the transposon-induced allele *lin-3(sy91::Tc1)* by mating *lin-3* males with hermaphrodites of the strain RW7096, which has high rates of transposition of the transposon *Tc1*¹⁶. *sy91* confers a recessive Vul phenotype (Fig. 3b) and was recovered because it fails to complement the paternal *lin-3* mutation. Genetic mapping showed that the *Tc1* insertion called *syP1* cosegregates with the mutation *sy91* (Fig. 2 legend). DNA sequences that flank *syP1* were used to identify the genomic clones λ PS#1 and λ PS#2. Two observations confirm that these genomic clones cover at least part of the *lin-3* locus. First, λ PS#1 detects the polymorphism *syP2* associated with the mutation *lin-3(n1058)* and which is located 2.5 kilobases (kb) from the *syP1* *Tc1* insertion. Second, both genomic clones can rescue the Vul phenotype of *lin-3* mutations when introduced as extrachromosomal multicopy transgenes. A 5-kb subclone called pRH9 and a 3.3-kb subclone called pRH19, both of which cover the location of *syP1* and *syP2*, can also rescue *lin-3*. pRH19 was used to isolate the complementary DNA clones pRH39 and pRH40. We determined the nucleotide sequences of the cDNA clone pRH39 (Fig. 4) and of the genomic region equivalent to pRH19 (data not shown). The two cDNAs appear to encode full-length Lin-3 proteins. The results of site-directed mutagenesis experiments

confirm that pRH39 and pRH40 are *lin-3* cDNAs. We introduced mutations into the genomic clone pRH9 that would change the sense of two codons of the transcription unit defined by the cDNAs (Fig. 4 legend). These mutations abolish the ability of this clone to rescue the Vul phenotype of *lin-3(n378)* when introduced as a transgene (Fig. 4 legend). Partial sequencing of *lin-3* genomic DNA indicates that the region of pRH39 and pRH40 that encodes the signal peptide is not located within pRH9 or pRH19. One explanation for the ability of these genomic clones to rescue *lin-3* is that they contain a genomic region that has characteristics of a 5' exon that could encode an alternative signal-peptide-like sequence (Fig. 4 legend). This region could be an alternative 5' end of the *lin-3* locus or a cryptic 5' end used only by the transgenes.

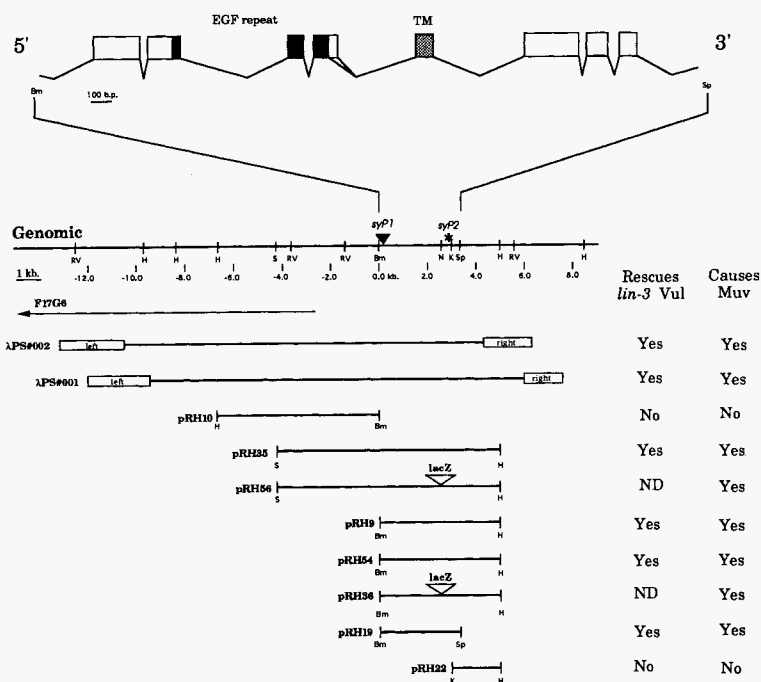
Lin-3 structure is similar to EGF-R ligands

The Lin-3 protein predicted from nucleotide sequence is a membrane-spanning protein of 438 or 423 amino acids with a single extracellular EGF repeat. Two forms of the protein are encoded by alternatively spliced transcripts and differ in the presence or absence of 15 amino acids between the EGF repeat and the transmembrane domain. The cytoplasmic domain of Lin-3 comprises 181 amino acids of novel sequence (Fig. 4). The EGF repeat of Lin-3 has statistically significant sequence similarity to many of the EGF repeats of other genes (Fig. 5). An EGF repeat is a motif consisting of six cysteine residues with semi-conserved spacing (reviewed in ref. 17). EGF repeats are present in a variety of proteins of many different functional classes^{17,18}. Lin-3 does not have overall sequence similarity to any characterized gene, and is most probably a new member of the EGF group of growth factors. The EGF repeat of Lin-3 has Tyr 13 and Arg 41, which are conserved preferentially among the EGF repeats used as growth factors (Fig. 5). Also, the overall structure of Lin-3 is most similar to the structure of the growth factor precursors, which beyond the presence of at least one EGF repeat and a transmembrane domain have little sequence similarity common to the entire class.

The EGF group of growth factors includes EGF itself^{19,20}, transforming growth factor- α (TGF- α)²¹, heparin-binding EGF²², amphiregulin²³ and vaccinia virus growth factor²⁴, which

FIG. 2 Molecular map of the *lin-3* locus. A physical map of the *lin-3* locus (centre) shows the position of the two *lin-3* restriction-fragment length polymorphisms (RFLPs) *syP1* and *syP2*. The ability of genomic subclones (bottom) to rescue a *lin-3* Vul phenotype and to cause a dominant Muv phenotype when introduced as transgenes is tabulated on the right. An expanded view of the *lin-3* transcription unit (top) is shown with exons represented as boxes: solid black, EGF repeat; stippled, putative transmembrane domain (TM). The position of *lin-3* exons outside of the 3.7 kb of sequenced genomic DNA are not determined. Left corresponds to left on the genetic map of LG IV. The unique *Bam*HI site is defined as position +1 of *lin-3* genomic DNA. The restriction map of K, N, S and Sp sites is complete only from +0 kb to +5 kb (abbreviations: K, *Kpn*I; N, *Nde*I; S, *Sac*I; Sp, *Spe*I; Bm, *Bam*HI; RV, *Eco*RV; H, *Hind*III; ND, not determined).

METHODS. Transposon tagging of *lin-3*: PS231 [*lin-3*(e1417) *dpy-20*(e1362)/*lin-3*(e1417) *unc-31*(e169)/*him-5*(e1490)/] males were mated with hermaphrodites of the mutator strain RW7096 [*mut-6*(st702) *unc-22*(st192::Tc1)/*IV*]¹⁶ and the progeny were examined for Egl non-Unc-22 animals. A single mutation, *lin-3*(*sy91*::Tc1), was found among 10,000 hermaphrodite cross progeny. *sy91* confers recessive Vul and Egl phenotypes (see Fig. 3b). Mapping of *syP1*: the Tc1 transposon insertion *syP1* was shown to map within a 95% confidence interval of 0.046 map units (mu) to the left of *lin-3* and 0.076 mu to the right of *lin-3* using a genetic methodology similar to that of Ruvkun *et al.*⁴⁰. *syP1* is a novel band of 2.0 kb detected on Southern blots^{39,41} hybridized with Tc1 DNA (pTR14, a gift from P. Anderson). Direct sequencing demonstrates that *syP1* is a Tc1 insertion within a target site duplication of the sequence TA located at positions 243–244 of genomic sequence in an intron located between nucleotides 216 and 217 of cDNA sequence (Fig. 4). Isolation of *lin-3* genomic clones: The sequence flanking the *syP1* Tc1 insertion was amplified from *Xba*I-digested *lin-3*(*sy91*) genomic DNA using inverse polymerase chain reaction (PCR)⁴². Digested genomic DNAs were circularized by self-ligation at a concentration of 7.5 µg ml⁻¹ with T4 DNA ligase (New England Biolabs) at 1 U per µl at 15 °C for 16 h, concentrated and desalted. PCR was according to the standard protocol of Perkin Elmer/Cetus (Norwalk, Connecticut) with the following modifications: circularized genomic DNA was used at 7 µg ml⁻¹ and a single primer (5'-ACCAAAAGTGGATATCTTTTGCCAGC-3') specific to the inverted repeat of Tc1⁴³ at 2 pmol µl⁻¹. A subcloned 400-bp product of the PCR detects a 400-bp *Xba*I fragment in non-*lin-3*(*sy91*) strains and a 2.0-kb *Xba*I fragment in *lin-3*(*sy91*) strains and was used to isolate λPS#1 and λPS#2 from a λ2001 genomic library⁴⁴. Identification of *syP2*: λPS#1 was used to search for additional *lin-3* RFLPs in genomic DNAs from strains carrying one of the following *lin-3* alleles: e1417 (ref. 5); n378, n1058, and n1059 (ref. 6); s751 and s1263 (ref. 45); sy51, sy52, and sy53 (R.J.H. and P.W.S., unpublished); sy91 (this study). The RFLP *syP2* is present in DNA from a *lin-3*(n1058) strain and consists of a novel *Eco*RV site at position +2.8 kb (data not shown). Transgenic rescue of *lin-3*: transgenic animals were created using the technique of Mello *et al.*³³. The smallest identified genomic region sufficient for transgenic rescue is the 3.3-kb insert of pRH19. All clones that can rescue the Vul phenotype of *lin-3* can also cause a



dominant Muv phenotype. pRH9(SK+) and pRH54(pMob KS) contain the same 5-kb *lin-3* region ligated to different vector sequences, suggesting that the adjoining vector sequence is not aiding the rescue. Each clone was injected into 6–20 animals and all the F₁ progeny of injected animals and all of the progeny of F₁ transgenic animals were examined. At least one stable line was analysed for each clone. λPS#1, λPS#2, pRH9, pRH19, pRH22 and pRH10 were each separately injected at a concentration of 50 ng µl⁻¹ with 50 ng µl⁻¹ of pRF4 (ref. 33), which causes a dominant Rol phenotype, into *lin-3*(e1417) *unc-22*(e66)/++ hermaphrodites. Unc-22 Rol progeny from injected animals were scored for rescue. For pRH19, 2 stable lines segregated Rol Uncs 19% of which were Egl, 78% wild type, and 3% Muv (*n* = 68). For pRH9, 25/131 Rol F1 were Muv and 5/5 stable lines gave frequent Muv progeny. For pRH22, 0/65 F1 Rol were Muv and stable lines segregated Rol Uncs, 91% of which were Egl, 9% wild type and 0% Muv (*n* = 35). pRH10 segregated no Muv progeny in 2 stable lines. e1417 homozygotes are 87% Egl⁶. pRH9 was also injected at 50 ng µl⁻¹ with the cosmid C14G10 (which contains wild-type *unc-31* DNA; R. Hoskins, personal communication) at 20 ng µl⁻¹ into *lin-3*(+) *unc-31*(e169) animals. This injection yielded the reference transgene syEx13 that was used in the ablation and epistasis experiments. The following injections also generated stable lines that segregated Muv progeny: pRH9 at 50 ng µl⁻¹, pRF4 at 50 ng µl⁻¹; pRH35 at 75 ng µl⁻¹, pRF4 at 50 ng µl⁻¹; pRH54 at 42 ng µl⁻¹, pRF4 at 50 ng µl⁻¹, pSK+ at 22.5 ng µl⁻¹; all into *lin-3*(n378) animals that were suppressed for their Egl phenotype by passage through the dauer stage⁶.

lin-3 has diverged from a common ancestor of the diverse mammalian growth factors. By RDF2 analysis²⁹, the EGF repeat of Lin-3 is most similar to the EGF repeats of the growth factors from myxoma virus (MVGF)³⁰ and Shope fibroma virus (SFGF)³¹. These two genes differ from the other EGF-like growth factors in that they lack possible membrane-spanning domains. MVGF has been shown to bind the EGF-R at a 200-fold lower affinity than EGF itself³², but the function of SFGF is unknown.

lin-3 transgenes cause excess vulval induction

Transgenes containing wild-type *lin-3* DNA confer a dominant multivulva (Muv) phenotype (Fig. 3c) in which up to all six of the VPCs have vulval fates. The transgenes are created by a method that concatenates about a hundred copies of the injected DNA into an extrachromosomal array³³ and thus are likely to express levels of a Lin-3 protein that are higher than the levels of Lin-3 expressed in a wild-type animal. We believe the transgenes cause excess vulval induction because the process of vulval induction is sensitive to overexpression of Lin-3. In contrast,

are known ligands of the EGF-R, as well as rat *neu* differentiation factor²⁵ and the human heregulins²⁶, which are ligands of *neu*/HER2, one of two other members of the EGF-R subfamily. The membrane-proximal EGF repeat is known in several cases to be the receptor-binding domain of these growth factors. Most of these molecules can undergo proteolytic processing to release this EGF repeat as a secreted molecule. In the case of TGF-α, the unprocessed membrane bound form can also activate the receptor (reviewed in ref. 27). Processing of Lin-3 has not been demonstrated. The alternative versions of the Lin-3 protein differ in the region between the EGF repeat and the membrane-spanning domain where processing would have to occur. The different Lin-3 proteins could differ in their ability to be processed. The use of alternative splicing to produce membrane-bound ligands that differ in their ability to be processed has been demonstrated for the Kit-ligand²⁸, but has not been seen with the EGF group of growth factors.

The phylogenetic relationship of *lin-3* to the EGF group of growth factors remains unclear. *lin-3* is not a clear homologue of any one member of the group and a simple hypothesis is that

mutations that lower *lin-3* activity decrease the number of VPCs that have vulval fates⁵⁻⁸. Thus, the amount of Lin-3 protein produced may control the number of VPCs that have vulval fates.

The *lin-3* transgenes act via *let-23*

We ordered the action of *lin-3* before the action of *let-23* based on a test of genetic epistasis using the dominant Muv phenotype of the *lin-3* transgenes. The *let-23* mutation *sy97* causes a highly penetrant Vul phenotype such that more than 99% of *sy97* homozygotes fail to lay eggs (the Egl phenotype)⁹. Ninety per cent of animals bearing the transgene *syEx13*, which contains wild-type *lin-3* DNA, are Muv. *let-23(sy97)* homozygotes carrying the transgene *syEx13* are not Muv and 99.7% are Egl (see Fig. 3 legend). Therefore the *lin-3* transgenes can only stimulate vulval fates if *let-23* is functional and thus *lin-3* is likely to be an upstream activator of *let-23*. Previous experiments in which Vul mutations of *lin-3* and *let-23* were indirectly ordered also suggest that *lin-3* acts before *let-23* (ref. 7). In contrast to the behaviour of the *lin-3* transgenes, multicopy *let-60* transgenes cause a Muv phenotype even in a *let-23(sy97)* mutant¹².

Anchor cell expression of *lin-3::lacZ*

To determine where the *lin-3* transgenes are expressed, the *lacZ* gene of *Escherichia coli* was inserted in-frame into the first cytoplasmic exon of *lin-3* (Fig. 6). The *lin-3::lacZ* fusion should produce a protein consisting of the extracellular and transmembrane domains of *lin-3* followed by a β -galactosidase domain encoded by *lacZ*. In transgenic animals this fusion produces

detectable β -galactosidase activity while maintaining the ability to stimulate excess vulval development. pRH36 contains the *lin-3::lacZ* fusion within a 5-kb *lin-3* genomic region equivalent to pRH9, whereas pRH56 contains an additional 4.6 kb of 5' genomic sequence (shown in Fig. 2). A transgene derived from pRH36 shows specific expression of β -galactosidase in the anchor cell. This expression is detectable during the lethargus between the L2 and L3 larval stages (Fig. 6a, b) and remains detectable in the L4 stage when vulval morphogenesis is nearly complete (Fig. 6d). Ablation experiments indicate that vulval induction occurs late in the L2 lethargus or early in the L3 (ref. 2) and thus the transgene is expressed before or during the time of vulval induction. pRH56 directs other localized expressions (data not shown), as would be expected, given that *lin-3* has other developmental phenotypes⁶. Nonetheless, the only expression near the VPCs is in the anchor cell. The expression patterns of pRH36 and pRH56 suggest that the 5-kb *lin-3* region represented by pRH9 could be a minimal region sufficient for vulval rescue which might not contain all control sequences required for other expressions. As the pRH36 transgene can stimulate excess vulval development (Fig. 6d) while displaying specific expression in the anchor cell, we infer that the anchor cell expression is relevant for vulval development.

lin-3 transgenes may act in the anchor cell

To test whether the ability of the transgenes to cause a Muv phenotype was dependent upon the inductive signal, we ablated the gonad precursors, and hence the anchor cell, in early L1

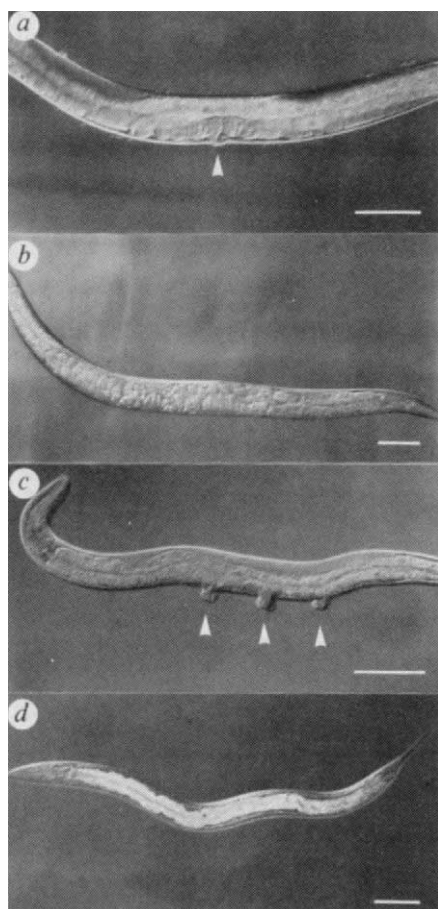


FIG. 3 Nomarski photomicrographs of *lin-3* phenotypes. a, An adult wild-type *C. elegans* hermaphrodite. The position of the vulva is marked by an arrowhead. The vulva is necessary for copulation with males and for egg laying. 20 $\times$ Neofluor objective. b, An adult vulvaless *lin-3(sy91::Tc1)* hermaphrodite. Mutations that lower *lin-3* activity, like *sy91*, result in decreased

levels of vulval induction. The lack of a vulva prevents egg laying, but does not prevent internal self-fertilization². The individual shown has no vulva and is retaining late stage embryos indicating that it has an egg laying defect (Egl). 10 $\times$ Neofluor objective and 1.25 $\times$ optovar. c, A young adult multivulva *syEx13* hermaphrodite. The transgene *syEx13* is an extrachromosomal array containing multiple copies of wild-type *lin-3* and *unc-31* DNA. The transgene causes excess vulval induction, which we believe is due to overexpression of Lin-3. Ectopic pseudovulvae are often visible as bumps on the ventral side of an adult worm when more than the wild-type number of VPCs assume vulval fates^{5,8}. The animal shown has three pseudovulvae which are marked by arrowheads and has a phenotype common for the transgenic *lin-3* Muv animals. 20 $\times$ Neofluor objective. d, An adult, gonad-ablated *syEx13* animal. Ablation of the gonad precursors, which prevents the development of the anchor cell, in *syEx13* animals lowers the ability of the transgenes to stimulate vulval induction such that most ablated animals are Vul like the one pictured. The ablation also destroys the germ line. Control mock-ablated *syEx13* animals had an average of 143% induction. Seven animals had wild-type vulval development of P[5-7].p, one animal each had P[4-7].p, or P[4-7].p and P8.pa, or P[4-8].p forming vulval tissue, and five animals had six VPCs forming vulval tissue. Ablated *syEx13* animals had an average induction of 23%. 12 animals had no induced VPCs, in 2 animals P[3-5].p developed as vulval tissue, and in one animal P4.p, P5.pp, and P[6-8].p developed as vulval tissue. 10 $\times$ Neofluor objective. Scale bars in a-d, 100 μ m.

METHODS. Ventral is down in all photographs and anterior is left in a, c and d. All animals except for the one in c are anaesthetized⁴⁶. Ablation of the four gonad precursors Z1-Z4 of *unc-31(e169); syEx13* L1 hermaphrodites was done as described^{34,46,47}. The extent of vulval differentiation is defined in ref. 9 such that the extent of vulval development can range from 0% when no VPCs have vulval fates per animal, to 200% when 6 VPCs have vulval fates per animal. Because the *syEx13* transgene can be lost mitotically, only animals that were non-*Unc-31* through the L4 stage were included in the analysis. Any individual non-*Unc-31* animal could lack the *syEx13* transgene in the appropriate tissue for vulval induction. The proportion of mock-ablated, non-*Unc-31* *syEx13* animals that have wild-type patterns of vulval development should represent a maximal estimate for the frequency of such chimaerism. The epistasis of *syEx13* and *let-23(sy97)* was tested by constructing a strain of the genotype *let-23(sy97) unc-4(e120); unc-31(e169); syEx13*. Non-*Unc-31* hermaphrodites were picked as L4s and rescored as adults for their Muv and Egl phenotypes; 89% of L4 non-*Unc-31* progeny from an *unc-31(e169); syEx13* mother were Muv as adults ($n=100$). No L4 non-*Unc-31* progeny from a *let-23(sy97) unc-4(e120); unc-31(e169); syEx13* mother were Muv as adults and only one was capable of laying eggs ($n=348$). *let-23(sy97)* animals are Egl⁹. Strains and routine genetic manipulations are as described in ref. 48.

AAGTTT

8 AGATTGTACATAGCCATCGGCACCATCGCCACTCAACGGGTATTCAATGCTGAAAT

67 TTAATAACGGATCGTCGGCTTGTCTGGTGTTCTTCCACCCTTATGCATCAATTTTTCC

126 TGTTTCATAAGATATCGTCGCCACAAGTTTCCAAAAATG CGG AAA ATG CTA CTT
Met Arg Lys Met Leu Leu

180 TTT TGC ATC CTT CTA CTC TTT ATG CCT CAA TTT ACA GTT TCA GAA
7 Phe Cys Ile Leu Leu Leu Phe Met Pro Gln Phe Thr Val Ser Glu

225 TCG TGT CTC CCT TCG TGG TTT CGT CAA GAA CGT AGT GCT CCC GAA
22 Ser Cys Leu Pro Ser Trp Phe Arg Gln Glu Arg Ser Ala Pro Glu

270 CAG CTT CAA TCT GCA GAG AAT GCA GCT GAA AAT AGT GGC TCT GTA
37 Gln Leu Gln Ser Ala Gln Asn Ala Ala Gln Asn Ser Gly Ser Val

315 GCA CCC GAT ACT TCT CGA AAT TCT CTA GAA ACA AAC GAA ATA GGT
52 Pro Pro Asp Thr Ser Arg Asn Ser Leu Glu Thr Asn Glu Ile Gly

360 GAT GCA CCG TCG TCG ACT TCG ACA CCT GAA ACA CCT ACT GAA ACT
67 Asp Ala Pro Ser Ser Thr Ser Thr Pro Glu Thr Pro Thr Glu Thr

405 ACG ATT TCC GAA GCT GGA GAC GAT GAA AAA CGA ACT GAA GAG GTT
82 Thr Ile Ser Glu Ala Gly Asp Asp Glu Lys Arg Thr Glu Glu Val

450 GCA AAA GAA TTA ATC GAG AAA GAA GCA GAA TAT GAG GGT GAA TAT
97 Ala Ala Gln Leu Ile Glu Lys Glu Ala Glu Tyr Glu Gly Glu Tyr

495 GAA GAT GAA AAG GTT GAT GAA GAA GTA GAA GAA GCG TTA AAA TAT
112 Glu Asp Glu Lys Val Asp Glu Glu Val Glu Glu Ala Leu Lys Tyr

540 AAT GAA GAT GCC ACT CAA GAT GCC ACG TCG ACT CTT AAA CCG GCG
127 Asn Glu Asp Ala Thr Gln Asp Ala Thr Ser Thr Leu Lys Pro Ala

585 GTT CGG AAG GAA ATC GAG AAG TTG AAA GAA GCA AAA TGC AAA GAC
142 Val Arg Lys Glu Ile Glu Lys Leu Lys Glu Ala Lys Cys Lys Asp

630 TAC TGT CAT CAC AAC GCG ACA TGC CAC GTG GAA GTG ATA TTC CGT
157 Tyr Cys His His Asn Ala Thr Cys His Val Glu Val Ala Phe Cys

675 GAA GAT AGA GTT TCA GCA GTT GTT CTT TCT TGC CAT TGT CCA CAG
172 Glu Asp Arg Val Ser Ala Val Val Pro Ser Cys His Cys Pro Gln

720 GGT TGG GAA GGC ACT CGT TGT GAT CGT CAC TAC GTT CAG GCG TTC
187 Gly Trp Glu Gly Thr Arg Cys Asp Arg His Tyr Val Gln Ala Phe

765 TAT GCC CCA ATC AAC GGC AGA TAT AAT GTA CGT TTG AGC AGC ATG
202 Tyr Ala Pro Ile Asn Gly Arg Tyr Asn Val Arg Leu Ser Thr Met

810 AGC AGC ACG GCG CAA CTC CTC GTT CAA CAA TCT TCA ACA TCA GCT
217 Ser Ser Thr Cys Gln Leu Leu Val Gln Gln Ser Ser Thr Ser Ala

855	ATT	CCT	GCG	TTC	GCA	TTT	CTG	ATT	GTC	ATG	CTC	ATC	ATG	TTT	ATA	
232	Ile	Pro	Ala	Phe	Ala	Phe	Leu	Ile	Val	Met	Leu	Ile	Met	Phe	Ile	
900	ACA	ATT	GTT	GTT	TAT	GCT	TAT	AGA	AGA	ATG	TCT	AAA	CGA	TCG	GAT	
247	Thr	Ile	Val	Val	Tyr	Ala	Tyr	Arg	Arg	Met	Ser	Lys	Arg	Ser	Asp	
945	GAT	ATG	ACA	TAT	ACA	ATG	ATG	CAT	ATG	TGC	CCA	CCA	GAA	GCA	TTC	
262	Asp	Met	Thr	Tyr	Thr	Met	Ser	His	Met	Cys	Pro	Pro	Glu	Ala	Phe	
990	AAT	GTC	CTC	AAA	ACA	CCA	AAT	GGA	CGA	CAT	ATT	CCA	GTT	CAT	CAA	
277	Asn	Val	Leu	Lys	Thr	Pro	Asn	Gly	Arg	His	Ile	Pro	Val	His	Gln	
1035	ATT	CCA	TCA	TGT	TCT	TAT	ACT	ATC	CCA	ACG	GGT	ACA	GTA	CCT	CTT	
292	Ile	Pro	Ser	Cys	Ser	Tyr	Thr	Ile	Pro	Thr	Pro	Gly	Thr	Val	Pro	
1080	CCA	AAT	ATA	TCA	TCA	ACT	CCT	GGA	TCA	AGA	ATA	CCC	ACT	CGT	CAA	
307	Pro	Asn	Ile	Ser	Ser	Thr	Pro	Gly	Ser	Arg	Ile	Pro	Thr	Arg	Gln	
1125	CAA	GCT	ATT	CGA	AAT	AAT	GAA	CAA	GCA	CGG	AAC	AAC	TTT	TTC	AGC	
322	Gln	Ala	Ile	Arg	Asn	Asn	Glu	Gln	Ala	Arg	Asn	Asn	Phe	Phe	Ser	
1170	ATT	CTC	AGA	AGT	CAA	GGT	ACC	ATT	CCA	TCC	AGG	AGT	ATC	AAT	GAC	
337	Ile	Leu	Arg	Ser	Gln	Gly	Thr	Ile	Pro	Ser	Arg	Ser	Ile	Asn	Asp	
1215	GAC	GAC	ACG	CCG	AAG	CAC	TAC	AAG	TCA	GTG	CCA	CGT	GTT	GAA	GTT	
352	Asp	Asp	Thr	Pro	Lys	His	Tyr	Lys	Ser	Val	Pro	Arg	Val	Glu	Val	
1260	TCA	GCA	ATT	AAT	TAC	TCT	GGC	CAC	ATT	GAT	TTT	TCA	ACA	GTA	TCA	
367	Ser	Ala	Ile	Asn	Tyr	Ser	Gly	His	Ile	Asp	Phe	Ser	Thr	Val	Ser	
1305	TAT	CAG	TCG	ACT	GAA	TCA	GAA	GTT	TCA	AAA	GCA	TCA	GTA	ACA	TGT	
382	Tyr	Gln	Ser	Thr	Glu	Ser	Glu	Val	Ser	Lys	Ala	Ser	Val	Thr	Cys	
1350	CCA	CCA	CCG	GCG	CAC	ACT	GTA	ATT	AAT	ATC	GAG	TTG	GAT	TCT	GCA	
397	Pro	Pro	Pro	Ala	His	Thr	Val	Ile	Asn	Ile	Glu	Leu	Asp	Ser	Ala	
1395	GAT	ACG	AAT	TTT	CGA	TCC	CCG	TCT	CGA	AGT	TCT	GGA	GAA	CAA	GGA	
412	Asp	Thr	Asn	Phe	Arg	Ser	Pro	Ser	Arg	Ser	Ser	Gly	Glu	Gln	Gly	
1440	TCA	CCA	GCA	ACA	TGT	GAA	CCA	ATG	ATT	CGA	CAC	ACA	TGAAAT	TATAA		
	Ser	Pro	Ala	Thr	Cys	Glu	Pro	Met	Ile	Arg	His	Thr	End			
1487	TTTGTTTCCCTTATTTTGTGCTTATCTTTTTCTTTTTTCATCAACTCTCTGTGCTATAAT															
1546	TGTGATTTACCACATGCTCAAACATCTCTGGTCTCTTCAAATTTTTTCGCTTTCTCTTTTT															
1605	TTCCCTCTATTTTTCATATCGTTCTACATGTTTTATAGATTGCATTGGCACCCCTATTATA															
1664	TCCCACCCCAATGTTCTATAGTTCTCTCACTCTCCCTCTCAGTTCCTGTGACAATTTGT															
1723	ATAATTTTGGCAGTTCAACTCTCGATTTCACGGGTACAATATTTCATCGTCTTTTTG															
1782	TAATTTCCTTTGTATCCCTAGTCTCTTCAGGTCCTCGTTGAAAAAACTAAACATTTATTT															
1841	CATGTTTTTATTAATGGAAAAATGAGAAAAATCATATAG															

FIG. 4. Sequence analysis of *lin-3*. A composite sequence of the *lin-3* cDNAs and the deduced Lin-3 protein is shown with key features noted. An amino-terminal hydrophobic stretch predicted to be a signal peptide, and a central hydrophobic stretch predicted to be a transmembrane domain are underlined. The EGF repeat is boxed. Sequences similar to the sites at which EGF and TGF- α are processed⁴⁷ are overlined where present in the extracellular domain of Lin-3. A stretch of 15 amino acids encoded by an alternatively spliced region of RNA is underlined in bold. A potential extracellular N-glycosylation site is located at position 161. Vertical arrowheads show positions of known introns. The region of the Lin-3 protein after the signal peptide and before the EGF repeat is rich in the amino acids proline, glutamate, serine and threonine and is thus formally similar to PEST-sequence domains that are proposed to mark intracellular proteins for rapid turnover⁴⁹. Translation is predicted to start at position 162 because of the location of the leader sequence and because of the presence of an upstream in-frame termination codon at position 60. cDNA sequence from position 217 to 1,400 is present within the genomic sequence of pRH19 and thus the cDNA sequence from +1 to +216, which encodes the signal peptide of the protein predicted above, is located to the left of position +1 in Fig. 2. One possible reason that genomic clones lacking this 5' region can provide *lin-3* function is the presence of a potential alternative 5' end located at position +196 to +248 of genomic DNA with the sequence 5'-TAAATGTTTCGGTAAATCGATTCTCTGAACGACTTCTAGTCGCAATTTGGTATGTC-3'. The use of the splice donor consensus at position 239 to 248 would splice nucleotide 241 of this genomic region to nucleotide 217 of the cDNA sequence (located at position +299 in genomic DNA). This splice would encode Och-Met-Phe-Gly-Lys-Ser-Ile-Pro-Glu-Arg-Leu-Leu-Val-Ala-Phe-Val and then continue with the protein predicted above, starting at Ser 20. The nucleotide sequence is a composite of the cDNAs pRH39 and pRH40. pRH39 starts at position 117 and ends at position 1844. pRH39 contains the alternatively spliced region of 792 to 836; pRH40 lacks this region.

METHODS. We determined the genomic sequence from the *Bam*HI site at position +1 to position +3,734 (data not shown), which is inclusive of the

minimal region sufficient for transgenic rescue. The sequence of the cDNA clone pRH39 has been determined for both strands except for nucleotide 353, which is only determined for the sense strand. Only the unique regions of pRH40 have been sequenced. Double-stranded DNA-sequencing reactions were done on CsCl-banded plasmid DNA using Sequenase v2.0 and other reagents from US Biochemical, with standard protocols⁴¹. *lin-3* sequence was analysed with multiple programs^{50,51}. Genbank v70.0 was searched with the BLAST program⁵² at the National Center for Biotechnology Information. The EGF repeat was first detected with the program MacPattern (R. Fuchs, personal communication). The statistical significance of regions of sequence similarity found by data base searches was assessed by RDF2 analysis²⁹. Mutations were introduced into pRH9 to change the third and/or fifth cysteine residues of the EGF repeat to serine residues as described in ref. 53 (reagents from Promega). This would block the formation of two of the three predicted disulphide bonds of the EGF repeat. The introduction of both mutations completely blocked the ability of derived transgenes to rescue the Egl phenotype of *lin-3* and to cause a Muv phenotype (below). Both single mutations contribute to this effect, suggesting that the transgenes produce an EGF repeat protein that is providing the *lin-3* function. Oligo Cys 3 (5'-ACTTCCACGTGGGACGTCGCGTTGTGA-3') changes nucleotide 650 to G and nucleotide 652 to C, which introduces a novel *AatII* site, causes a silent change in Thr 163, and changes Cys 164 to Ser. Oligo Cys 5 (5'-TTCCCAACCCTGCGGAGAAGTCAAAAAG-3') changes nucleotide 712 to C and nucleotide 716 to G, which destroys a *BstXI* site, causes a silent change in Pro 185 and changes Cys 184 to Ser. The plasmid pRH41 contains Ser 164, pRH42 contains Ser 184, and pRH43 contains Ser 164 and Ser 184. Transgenic animals were created as described in Fig. 2 by microinjecting a *lin-3* plasmid at 50 ng μl^{-1} and pRF4 at 50 ng μl^{-1} into dauer-suppressed *lin-3(n378)*. The penetrance of *lin-3* phenotypes for Rol animals segregating from stable transgenic lines created with these plasmids are: pRH9, 22% Egl, 72% Muv ($n=50$); pRH41, 15% Egl, 69% Muv ($n=48$); pRH42, 65% Egl, 0% Muv ($n=95$); pRH43, 99% Egl, 0% Muv ($n=76$); 97% of *lin-3(n378)* hermaphrodites are Egl without any transgenes⁶.

		6	13	20	31	41		Z	R
<i>lin-3</i> 149	L	K	E	A	K	C	-----KDYCHHNATCHVEVIFREDRVSAVVPSCHCPQGWEGTACDRHYVQAFYAP		
hEGF	N	S	D	S	E	C	P L S H D G Y C L H D G V C M Y I E A -----LDKYACNCVVGVIIGERCQYRD L K W W E L R	2.9	53/70
mEGF	N	S	Y	P	G	C	P S S Y D G Y C L N G G V C M H I E S -----LDSTYTCNCVIGIYSQDRQCQTRDL R W W E L R	3.8	55/67
hTGF- α	V	V	S	H	F	N	D C P D S H T Q F C F H -GTCRFLVQ-----EDKPAACVCHSGYVGA R C E H A D L L A	4.2	60/59
HB-EGF 101	L	G	K	K	R	D	P C L R K Y K D F C I H -G E C K Y V K E -----LRAPSCICHPGYHGERCHGLSLPVE	4.9	62/57
Amp1 39	N	R	K	K	K	N	P C N A E F Q N F C I H -G E C K Y I E H -----LEAVTCKCKQQEYFGERCGEK	2.3	48/60
VVGF 38	D	I	P	A	I	R	L C G P E G D G Y C L H -G D C I H A R D -----IDGMYCRCSHGYTGIR C Q H V V L V D Y	4.9	65/63
SFGF 26	I	V	K	H	V	K	V C N H D Y E N Y C L N N G T C F T I A L D N -----VSITPFCVCRINYEGR C Q F I N L V T Y	7.3	75/56
MVGF 30	I	I	K	R	I	K	L C N D D Y K N Y C L N N G T C F T V A L N N -----VSLNPFCACHIN Y V G S R C Q F I N L I T I	6.4	71/62
NDF 175	G	T	S	H	L	I	K C A E K E K T F C V N G G E C F T V K D L S -----NPSRYLCKCQPGFTGARCTENVP MK V Q	4.0	55/54
hHRG- α	G	T	S	H	L	V	K C A E K E K T F C V N G G E C F M V K D L S -----NPSRYLCKCQPGFTGARCTENVP MK V Q	4.5	57/57

FIG. 5 Comparison of the EGF repeat of Lin-3 to other EGF repeats. The EGF repeat of Lin-3 is aligned with EGF repeats known or believed to be used as growth factors. Lin-3 matches the consensus sequence C-X-C-X5-G-X-R-C of EGF repeats. The EGF repeat of Lin-3 has conservation or conservative substitutions of many of the residues conserved in EGF repeats including: Tyr 13, Gly 39, Arg 41, Ala for Gly 18, Trp for Tyr 37, and Val for Leu 47. The spacing between the first and second, and the third and fourth cysteines of Lin-3 is unique. The EGF repeat of Lin-3, like repeats used as growth factors, lacks the consensus for high affinity Ca^{2+} binding of Asp/Asn 2, Asp/Asn 4, Asp/Asn 22, Tyr/Phe 29 and Glu 38⁵⁴. RDF2 analysis²⁹ indicates statistically significant sequence similarity between the EGF repeat of Lin-3 to EGF repeats found in growth factors and to EGF repeats found in non-growth factors. The results of the RDF2 (500 shuffles, ktup=1, uniform

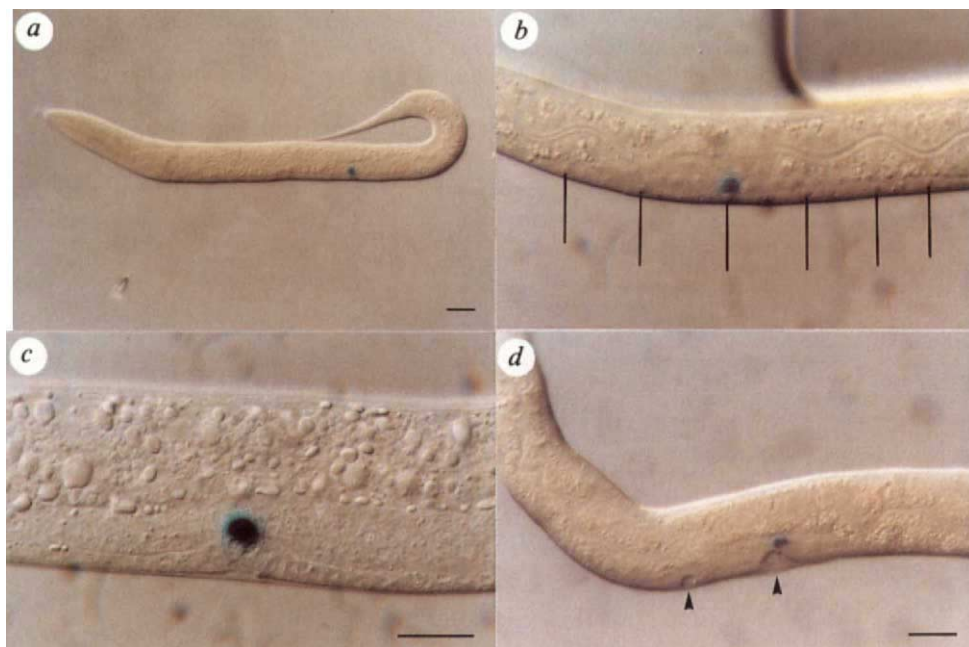
shuffling) are tabulated: z =(optimal score—the mean of the optimal alignment of shuffled sequences)/standard deviation of the optimal alignment of shuffled sequences; R is the ratio of the optimal alignment to the best optimal alignment of a shuffled sequence. Suggested minimal values for significance²⁹ are $z=3$ and $R=1$. Two examples of non-growth factor EGF repeats with high similarity to Lin-3 are: *crumbs* 426–465 (ref. 55; $z=4.6$, $R=62/60$) and *lin-12* 406–447 (ref. 56; $z=6.3$, $R=73/63$). References and abbreviations: hEGF (human epidermal growth factor)¹⁹, mEGF (murine EGF)²⁰, hTGF- α (human transforming growth factor- α)²¹, HB-EGF, (heparin-binding EGF)²², amph, (human amphiregulin)²³, VVGF (vaccinia virus growth factor)²⁴, SFGF (Shope fibroma virus growth factor)³¹, MVGF (myxoma virus growth factor)³⁰, NDF (rat *neu* differentiation factor)²⁵, hHRG- α (human heregulin α)²⁶. The numbering system of mouse EGF is used.

animals that carried *lin-3* transgenes. Gonad-ablated *syEx13* animals had an average of 23% induction compared with 143% induction in mock-ablated *syEx13* animals (see Fig. 3 legend for explanation of scoring). Specifically, 12 of 15 ablated animals

had no vulval differentiation and three animals had three to five VPCs generating vulval progeny. 7 of 15 mock-ablated *syEx13* animals had wild-type vulval induction and eight had vulval induction greater than wild-type (Fig. 3d). Therefore, the *lin-3*

FIG. 6 Nomarski photomicrographs of β -galactosidase expression in *lin-3::lacZ* transgenic animals. *a*, An L2 molt animal showing specific expression of β -galactosidase (blue) in the anchor cell. The old cuticle is being shed near the head and tail. 40 $\times$ Neofluor objective. *b*, A L2 molt animal with expression of β -galactosidase in the anchor cell. The six VPCs (P[3–8].p) are marked by vertical lines. Expression of β -galactosidase is detected before or during the time of vulval induction. 100 $\times$ Neofluor objective. *c*, β -galactosidase activity is still detected after VPC fates have been determined. In this animal the descendants of the VPCs are in the third and final round of division. At this stage the anchor cell is closely associated with the vulva and has a distinctive morphology. 100 $\times$ Neofluor objective. *d*, A Muv L4 hermaphrodite with staining of the anchor cell. All vulval divisions have been completed and vulval morphogenesis has started. The presence of two invaginations (marked by arrowheads) indicates that extra vulval induction has occurred. 40 $\times$ Neofluor objective and 1.6 $\times$ optovar. Ventral is down in all photographs and anterior is left in *a* and *d*. Scale bar, 20 μm .

METHODS. A *Sma*–*SpeI* fragment containing a *trpS::lacZ* fusion, a nuclear localization signal, and 3' untranslated sequence from *unc-54* was excised from the vector pPD16.51 (ref. 58) and end-filled. The 5.0-kb *lin-3* genomic *Bam*HI–*Hind*III insert of pRH9 was subcloned into pMob KS, digested with *Nde*I, which cuts at a unique site within the first cytoplasmic exon of *lin-3*, and end-filled. These two fragments were ligated together to create the plasmid pRH36. To construct pRH56, the *lin-3::lacZ* fusion of pRH36 was excised as a 9.5-kb *Bam*HI–*Hind*III fragment and subcloned into a *Hind*III–



*Bam*HI digest of a plasmid containing a *lin-3* genomic fragment from *Sac*I(–4.2 kb) to *Bam*HI(0.0 kb) in pMob KS. pRH36 was injected at 65 ng μl^{-1} and with pSK+ at 25 ng μl^{-1} and 10 ng μl^{-1} pMH86, which contains wild-type *dpy-20* DNA, into *dpy-20(e1282)* using the method described in Fig. 2. pRH56 was injected at 90 ng μl^{-1} with pMH86 at 10 ng μl^{-1} into *dpy-20*. β -galactosidase was detected by a standard protocol⁵⁸ for *c*, or by a modification (suggested by L. Carta) in which worms were fixed in 3.5% glutaraldehyde (EM grade 8% solution; Polysciences Inc.) for 15–30 min at 40 $^{\circ}\text{C}$ followed by staining at 40 $^{\circ}\text{C}$ for *a*, *b*, *d*.

transgene either acts in the gonad to stimulate vulval development, or acts elsewhere and requires the gonad to become active. Because *syEx13* contains the same *lin-3* genomic region as a *lin-3::lacZ* fusion that is expressed specifically in the anchor cell, we believe that *lin-3* acts in the anchor cell. These ablation studies do not indicate the site of action for the residual activity of the *lin-3* transgenes in the three ablated animals with vulval development. It is possible that the transgenes have erratic ectopic expression, or that the VPCs are responding to an overexpression of Lin-3 in other tissues that do not normally induce the vulva. We conclude the *lin-3* can act in a non-cell autonomous manner to stimulate vulval development.

lin-3 as inductive signal

We propose that *lin-3* encodes the vulval-inducing signal from the anchor cell defined by the ablation experiments of Kimble². This hypothesis is supported by several observations. First, the vulvaless phenotype of mutations that lower *lin-3* activity indicates that *lin-3* is normally necessary for the specification of vulval fates⁵⁻⁸. Second, presumptive overexpression of Lin-3 by multicopy transgenes increased levels of vulval induction. Together these two facts suggest that the dose of *lin-3* determines the extent of vulval development. Third, genetic epistasis experiments indicate that *lin-3* acts genetically upstream of *let-23* in a common pathway. Fourth, *lin-3* appears to be a new member of the EGF group of growth factors and *let-23* is homologous to the EGF receptor¹¹. Thus the molecular identities of *lin-3* and *let-23* and their genetic order of action suggest that *lin-3* could encode a ligand for the receptor encoded by *let-23*. Fifth, *lin-3::lacZ* fusions are expressed in the anchor cell at the time of vulval induction. Sixth, ablation experiments indicate that the *lin-3* transgenes can act in the gonad to stimulate vulval development. *lin-3* has the correct genetic properties, molecular structure and pattern of expression to be this inductive signal.

In addition to the inductive signal encoded by *lin-3*, two other intercellular signals have been proposed to regulate the fates of the VPCs. First, a lateral signal mediated through *lin-12* regulates the pattern of fates assumed by induced VPCs and appears to act after *lin-3* (refs 34-36). Second, an inhibitory signalling pathway defined by mutations in *lin-15* promotes epidermal

fates^{6,34,35,37}. The inhibitory signal may originate from the surrounding epidermis and thus would inhibit all the VPCs equally³⁸. Genetic epistasis experiments indicate that *lin-15*, like *lin-3*, acts to control *let-23* activity^{7,9} (L. Huang and P.W.S., unpublished results). Given that *lin-3* and *lin-15* are both regulators of *let-23*, it is possible that they either act in parallel and antagonistically on *let-23*, or that they act in series such that *lin-3* is a negative regulator of *lin-15*. We propose that *lin-3* and *lin-15* act in parallel on *let-23*. First, the nature of the *lin-3* and *let-23* proteins suggest Lin-3 could be a direct ligand of Let-23. Second, the inductive signal can influence the fates of VPCs in a *lin-15* mutant suggesting that the inductive signal does not require functional *lin-15* to influence the fates of the VPCs^{10,34}.

We propose that the VPCs possess a response pathway that originates with the receptor tyrosine kinase encoded by *let-23* and that acts through *let-60 ras* to specify vulval fates (Fig. 1b). In our view, the activity of this response pathway is controlled by two antagonistic upstream pathways. An inhibitory pathway mediated by *lin-15* lowers the basal activity of the response pathway in all VPCs in the absence of the inductive signal. We propose that *lin-3* is the inductive signal produced by the anchor cell and that binding of Lin-3 to Let-23 activates Let-23 and the response pathway. Because the inductive signal can apparently act at a distance⁴ Lin-3 may be proteolytically processed to release the EGF repeat as a secreted factor. It is possible though, that some wild-type induction uses a membrane bound form of Lin-3. For example, the VPC directly beneath the anchor cell, P6.p, could be induced by membrane-bound Lin-3 while the VPCs one cell distal to the anchor cell, P5.p and P7.p could be induced by secreted Lin-3.

The *lin-3* transgenes can stimulate all the VPCs in a given animal to have vulval fates. This observation confirms that each VPC is competent to respond to the inductive signal. Since this induction can occur when all six VPCs are present, we argue against the possibility that intracellular signalling among the VPCs has an absolute ability to limit the number of VPCs that differentiate as vulval tissue. Thus, during normal development the extent of vulval differentiation might be limited by the level of inductive signal. □

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Detection of TeV photons from the active galaxy Markarian 421

M. Punch^{*†}, C. W. Akerlof[‡], M. F. Cawley[§],
M. Chantell^{*}, D. J. Fegan[†], S. Fennell^{*†}, J. A. Gaidos^{||},
J. Hagan[†], A. M. Hillas[¶], Y. Jiang^{*}, A. D. Kerrick[#],
R. C. Lamb[#], M. A. Lawrence^{*}, D. A. Lewis[#],
D. I. Meyer[‡], G. Mohanty[#], K. S. O'Flaherty[†],
P. T. Reynolds[#], A. C. Rovero^{*}, M. S. Schubnell[‡],
G. Sembroski^{||}, T. C. Weekes^{*}, T. Whitaker^{*}
& C. Wilson^{||}

^{*} Whipple Observatory, Harvard-Smithsonian CfA, Box 97, Amado, Arizona 85645 USA

[†] Physics Department, University College Dublin, Belfield, Dublin 4, Ireland

[‡] Physics Department, University of Michigan, Ann Arbor, Michigan 48109, USA

[§] Physics Department, St. Patrick's College, Maynooth, County Kildare, Ireland

^{||} Physics Department, Purdue University, West Lafayette, Indiana 47907 USA

[¶] Physics Department, University of Leeds, Leeds LS2 9JT, UK

[#] Physics and Astronomy Department, Iowa State University, Ames, Iowa 50011 USA

PHOTONS of TeV energy have been observed from a few sources in our Galaxy, notably the Crab Nebula¹. We report here the detection of such photons from an extragalactic source, the giant elliptical galaxy Markarian 421. Mk 421 has a nucleus of the BL Lacertae type^{2,3}, and emission from it has been observed at radio⁴⁻⁶, optical^{3,6} and X-ray⁶⁻⁸ frequencies, and most recently in the MeV-GeV bands, by the EGRET detector aboard the Compton observatory⁹. In March-June 1992, we observed Mk 421 with the Whipple Observatory γ -ray telescope¹⁰, a ground-based detector that images Cerenkov light from air showers, and found a signal with statistical significance of 6σ above background. The flux above 0.5 TeV is 0.3 of that from the Crab Nebula. The source location agrees with the position of Mk 421 within the angular uncertainty (6 arc minutes) of the Whipple instrument. The fact that we have observed this relatively nearby source (redshift $z = 0.031$), whereas active galaxies and quasars that are brighter at EGRET energies but more distant have not been detected in the TeV energy range, may be consistent with suggestions^{11,12} that TeV photons are strongly attenuated by interaction with extragalactic starlight.

The very-high-energy γ -ray telescope¹⁰ at the Whipple Observatory images Cerenkov light from air showers on a two-dimensional array of 109 fast photomultipliers with a pixel size of 0.25° . Monte Carlo simulations^{13,14} and repeated observations of the Crab Nebula^{15,16} demonstrate that the Cerenkov light images of air showers induced by γ -rays can be reliably distinguished from those induced by cosmic-rays (that is, nucleons).

The most sensitive technique yet used by the Whipple group for this purpose ('supercuts'¹⁷) uses four parameters to characterize the roughly elliptical shower image. Two of these are the root-mean-square length and width of the ellipse. A third, 'distance', is the angular distance of the centroid of the shower image from the assumed source location in the image plane. A fourth parameter, 'alpha', gives the orientation of the image. Alpha is defined to be the angle between the major axis of the shower image and a line from its centroid to the assumed source location in the image plane. For γ -ray showers from a point-like source, alpha should be near 0° because the elliptical images point to the location of the source in the image plane. The supercuts procedure¹⁷ selects showers with small size, at distances from 0.51° to 1.1° , and with values of alpha $< 15^\circ$ degrees.

In Fig. 1a, the alpha distributions for on-source and off-source observations of Mk 421 are compared after the other supercuts selection criteria have been satisfied. For the region of alpha $< 15^\circ$ there is a 6.3σ excess, with 302 on-source showers and 166 off-source showers. These observations were made between 24 March and 2 June 1992 for a total of 7.5 hours on-source and an equal amount of time off-source. The excess corresponds to an average flux of 1.5×10^{-11} photons $\text{cm}^{-2} \text{s}^{-1}$ above 0.5 TeV, equivalent to 0.3 times that of the Crab Nebula. If one assumes isotropic emission at a distance of 124 Mpc, then the corresponding luminosity is $\sim 10^{43}$ erg s^{-1} . But as Mk 421 is known to show jet-like behaviour, the actual TeV luminosity may be considerably less.

For comparison, the alpha distributions for the previously reported observations of the Crab Nebula¹⁷ are shown in Fig. 1b. For the Crab Nebula the excess has a statistical significance of 34σ . For both sources, the data of Fig. 1 have been restricted to observations at elevations greater than 55° . The similarity between the Mk 421 excess in the small-angle region of Fig. 1 and the corresponding excess for the Crab Nebula corroborates the Mk 421 signal. As a measure of the stability of the Whipple detector, the on-source and off-source datasets contained 77,181 and 76,761 raw, uncut showers, respectively, a difference of only 0.55%. We have investigated the possibility that the excess shown in Fig. 1a may be a systematic effect related to the on- and

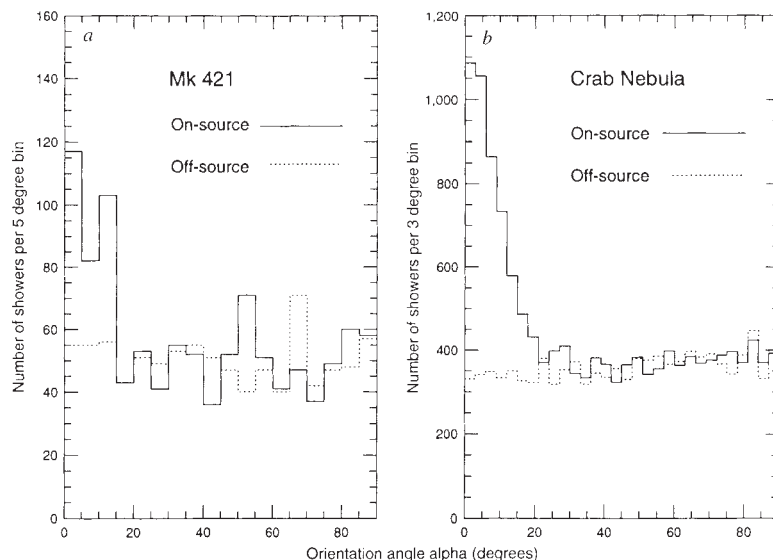


FIG. 1 On- and off-source orientation angle ('alpha') distributions for a, Mk 421 and b, Crab Nebula. The distributions are for those showers for which the other supercuts selection criteria¹⁶ have been satisfied. The supercuts selection value for alpha is 15° .

From the observations a two-dimensional map of the source region¹⁸ may be created. Figure 2 shows the map from the observations of Mk 421. The centre of the field of view corresponds to the known direction of the source. The peak seen is within 0.1° degree of this direction.

**James W. Kirchner*, Peter J. Dillon†
& Bruce D. LaZerte†**

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The theory tested here is a simple mechanistic method for predicting catchment response to changing acid anion concentrations directly from runoff chemistry⁸. The governing equations resemble those underlying many acidification computer models⁹⁻¹², but they are solved analytically, yielding catchment acidification response as a function of concentrations in catchment runoff. In a charge balance for typical acid-sensitive waters



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the left side expresses the total acid burden, and we hypothesize that the principal species of the right side are regulated, in the short term, by equilibria with solid, adsorbed and gas phases (for example by ion exchange, mineral dissolution or carbonate equilibria). In such heterogeneous equilibria, the concentrations C of any two ions i and j are related by the expression

$$(\gamma_i C_i)^{z_j} = K_{i,j} (\gamma_j C_j)^{z_i} \quad (2)$$

where γ_i and γ_j are single-ion activity coefficients, z_i and z_j are ionic valences, and the reaction constant $K_{i,j}$ subsumes the thermodynamic equilibrium constant, the activity of the non-aqueous phase and any intermediate reactions. If the solution composition shifts incrementally (and therefore activity coefficients and non-aqueous activities are constant), the relative shifts in the two concentrations will be

$$\frac{\partial C_i}{\partial C_j} = \frac{z_i}{z_j} \frac{C_i}{C_j} \quad (3)$$

We hypothesize that heterogeneous equilibria regulate the main species on the right-hand side of equation (1). We therefore rewrite (1) as

$$\Sigma \text{acids} = \sum_{i=1}^n z_i C_i \quad (4)$$

where Σacids is the sum of strong acid and organic anions. Differentiating equation (4), applying equation (3) throughout and rearranging terms yields

$$\frac{dC_j}{d\Sigma \text{acids}} = \frac{z_j C_j}{\sum_{i=1}^n z_i^2 C_i} \quad (5)$$

Given only the equilibrium concentrations of the n species on the right-hand side of equation (1), equation (5) predicts each ion's response to changes in Σacids . Equation (5) implies that shifts in Σacids will primarily be accommodated within the heterogeneous system by ions with high concentrations and large valences, not large shifts in minor ions.

Predicted responses for individual ions can be added⁸, yielding acidification responses for important water quality indicators, such as the sum of base cations (SBC)

$$\frac{d\text{SBC}}{d\Sigma \text{acids}} = \frac{4[\text{Ca}^{2+}] + 4[\text{Mg}^{2+}] + [\text{Na}^+] + [\text{K}^+] + [\text{NH}_4^+]}{\sum_{i=1}^n z_i^2 C_i} \quad (6)$$

the titratable acid neutralizing capacity¹³ (ANC)

$$\frac{d\text{ANC}}{d\Sigma \text{acids}} = \frac{d\text{SBC}}{d\Sigma \text{acids}} + \frac{9[\text{Al}^{3+}] + 6[\text{Al}(\text{OH})^{2+}] + 3[\text{Al}(\text{OH})_2^+] - 1}{\sum_{i=1}^n z_i^2 C_i} \quad (7)$$

and the molar sum of inorganic aluminium (Al_i)

$$\frac{d\text{Al}_i}{d\Sigma \text{acids}} = \frac{3[\text{Al}^{3+}] + 2[\text{Al}(\text{OH})^{2+}] + [\text{Al}(\text{OH})_2^+] + 2[\text{AlF}^{2+}] + [\text{AlF}_2^+]}{\sum_{i=1}^n z_i^2 C_i} \quad (8)$$

We used acid episodes observed during long-term monitoring studies^{14–16} in central Ontario to test whether equations (5)–(8) accurately predict catchment response to changes in acid loading. Acute droughts in the summers of 1987–90 permitted oxidation and mobilization of sulphur stored in catchment soils and wetlands¹⁷. In many catchments, autumn storms following summer droughts yielded streamflow with very high sulphate concentrations (Fig. 1). Acid anion levels remained high for months, falling throughout the year until the next summer's drought. Pronounced changes in base cation, hydrogen ion and

aluminium concentrations, as well as titratable alkalinity, accompanied the acid anion variations (Figs 1 and 2).

At 18 intensively monitored catchments spanning a range of run-off chemistries (Table 1), we averaged solute concentrations from stream samples taken before the 1987 drought, inserted the averaged values into equations (5)–(8), and compared the calculated estimates of catchment response with stream chemistry observed in the following four drought years (Fig. 2). This is a severe test for four reasons. First, unlike typical catchment acidification models, the theory contains no *ad hoc* empiricisms, arbitrary constants or adjustable parameters. Second, the chemical behaviour of catchment runoff before the first drought is very different from the behaviour against which the theory is tested (Fig. 1). Third, the type of input information

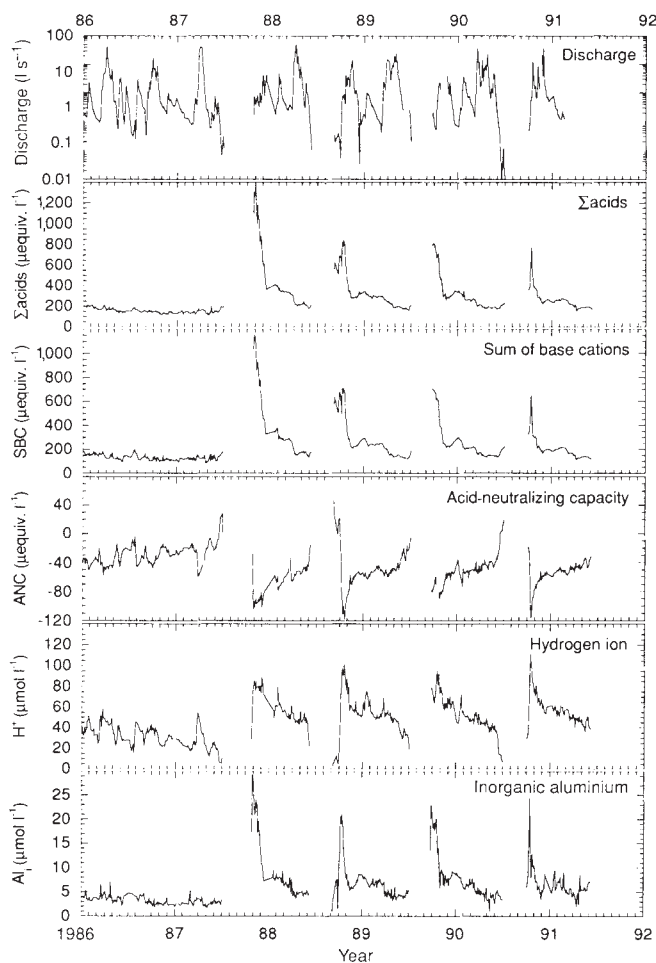


FIG. 1 Effects of summer droughts on stream discharge and chemistry at catchment 1, Plastic Lake, Ontario. Large data gaps indicate droughts (periods of no streamflow). Sampling interval for chemical data averaged 3 days, excluding drought gaps. Before the mid-1987 drought, runoff chemistry fluctuated within relatively narrow bounds. Droughts in four successive summers were followed by runoff with elevated concentrations of acid anions, base cations, hydrogen ions and inorganic aluminium, and sharply reduced acid neutralizing capacity. Each of these concentrations remained outside its pre-drought range for several months after each drought ended. Acid anion variations were dominated by sulphate; the sum of nitrate, chloride and organic acids averaged $70 \mu\text{equiv. l}^{-1}$, and never exceeded $250 \mu\text{equiv. l}^{-1}$, over the period shown. Runoff chemistry is not strongly correlated with streamflow over the period analysed here. Acid episodes are often driven by dilution effects or changes in flowpaths resulting from extreme hydrological events such as intense rainstorms or sudden snowmelt²⁸. Snowmelt episodes have been observed at some of our catchments during non-drought years²⁹, but during 1987–90 flow effects were dwarfed by the large changes in acid anion concentrations. In typical catchment data, where acid loading varies over a much narrower range, flow effects may obscure catchment response to acid anion variations.

(averaged concentrations) is distinct from the type of prediction (slopes of relative changes in concentrations). Fourth, the large changes in acid levels dwarf other sources of variation, so the data reflect catchment response to acid anions rather than to other factors (such as streamflow fluctuations).

Figure 2 compares acidification responses at two sites with the geochemical predictions derived from average pre-drought run-off chemistry. The lines shown are *a priori* predictions, not fitted relationships, so there are naturally some discrepancies with the data. Nonetheless, concentrations of base cations, hydrogen ions and aluminium generally respond to acid anion variations as predicted, and the theory predicts the observed differences in response between the catchments.

We constructed plots similar to Fig. 2 for all 18 study sites. We then compressed the results (Fig. 3), by comparing predicted slopes derived from pre-drought mean streamwater concentra-

tions (the solid lines in Fig. 2) with the least-squares regression slopes of all the data for each catchment. Equations (5)–(8) correctly predict both the magnitude of the average response across the 18 sites, and the differences in response between different sites (Fig. 3). When the 18 sites are ranked by the responsiveness of a given ion to acid anion concentrations, the rankings generally correspond to those predicted from pre-drought mean chemistry (Spearman $r_s > 0.65$, $p < 0.01$, for all ions in Fig. 3).

The large acid anion variations analysed here resulted from internal loading (changes in catchment sulphate storage) rather than external loading (changes in acid deposition rates). The prediction method tested here also explains catchment response to external loading⁸ at Birkenes¹⁸ (Norway) and Hubbard Brook¹⁹ (USA), where acid deposition has varied historically, and at Sogndal and Risdalsheia (Norway), where acid deposi-

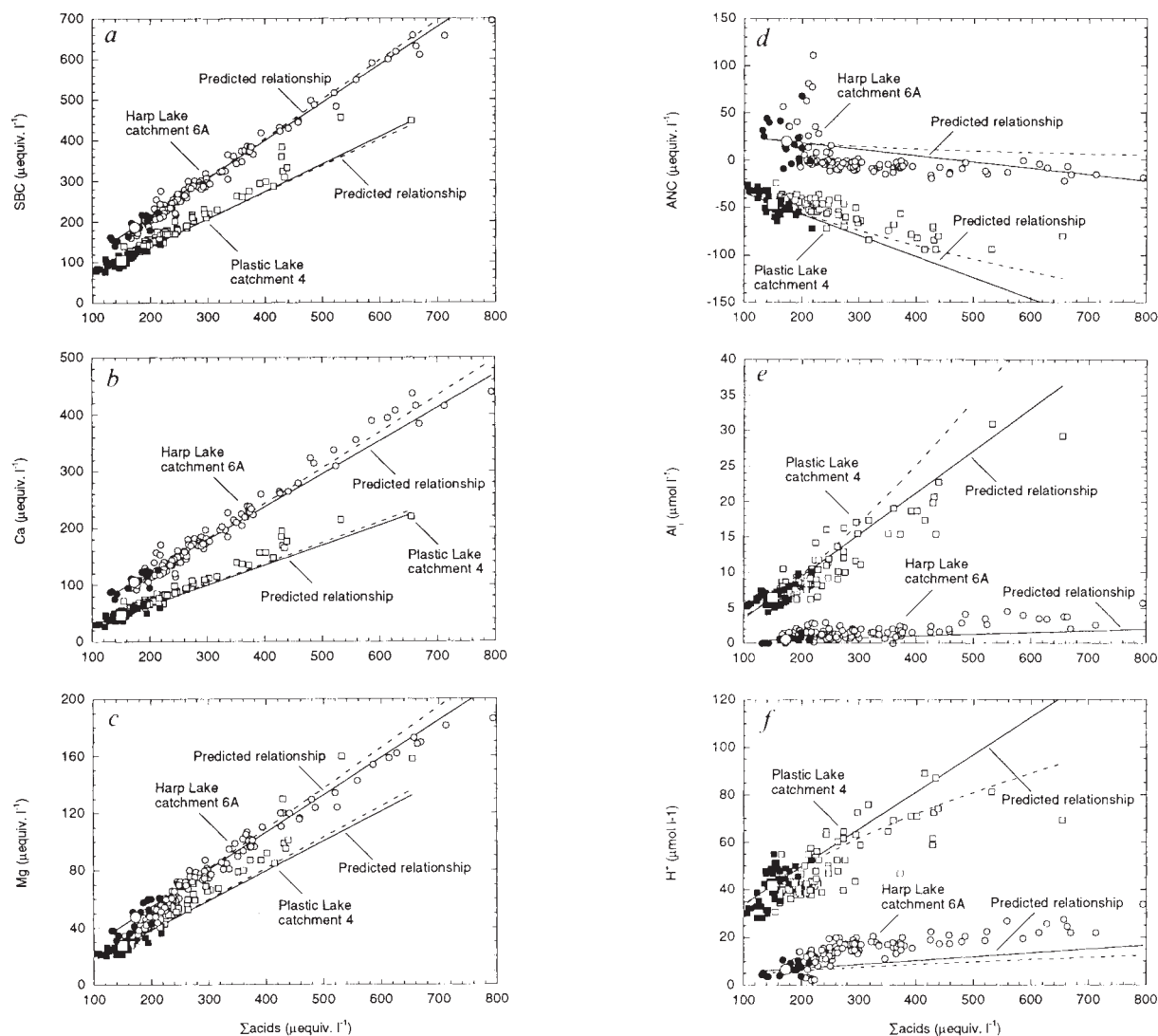


FIG. 2 Response of two monitored catchments to variation in acid anion concentrations (Σacids) resulting from summer droughts, compared to relationships predicted from equations (5)–(8) (solid lines) from averages (large open symbols) of samples taken before onset of 1987 drought (solid symbols). Panels show the effect of acid anion loading on (a) sum of base cations (SBC); (b) calcium concentrations; (c) magnesium concentrations; (d) acid neutralizing capacity; (e) inorganic aluminium concentrations; (f) hydrogen ion concentrations. All samples taken after the onset of the first drought (used to test, but not derive, predicted response) shown by small open symbols. Circumneutral Harp Lake tributary 6A and acidic Plastic Lake tributary 4 (see Table 1) drain forested catchments of 15 and 5 hectares, respectively. Linear extrapolations of slopes predicted from pre-drought

average concentrations (solid lines) are approximations to the nonlinear relationships (dashed lines) observed when equation (5) is integrated over large ranges of Σacids . Similarly, linear regression slopes plotted in Fig. 3 are approximations to the test data, which show some curvature, particularly for monovalent cations. The direction and approximate magnitude of the nonlinearities in the data can be predicted (J.W.K., manuscript in preparation) by differentiating equation (5). Data for extremely high acid anion concentrations ($\Sigma\text{acids} > 800 \mu\text{equiv. l}^{-1}$) have been omitted here, and omitted from regression relationships in Fig. 3; the response of some ions is markedly nonlinear above this range. Similar plots have been used to test computer simulation models of catchment acidification^{30,31}.

TABLE 1 Mean pre-episode runoff concentrations

	Plastic Lake catchment 4	Harp Lake catchment 6A	Range for all sites
Measured concentrations			
pH	4.38	5.17	4.20–5.48
ANC	–46	20	–71–80
SO ₄	73	101	25–147
NO ₃	0.5	6.4	0.1–8.0
Cl	14	12	8–30
Ca	47	106	42–132
Mg	28	48	23–69
Na	24	28	16–43
K	6.4	5.1	2.2–14.9
NH ₄	0.3	0.6	0.2–11.0
Al _{org} (μmol l ^{–1})	10.3	3.5	2.4–10.3
Al _i (μmol l ^{–1})	6.5	0.5	0.2–6.8
F _T (μmol l ^{–1})	3.7	2.1	2.1–4.0
Si (μmol l ^{–1})	129	96	47–129
DIC (mg l ^{–1})	2.0	3.0	0.8–6.0
DOC (mg l ^{–1})	15	9	3–21
Calculated speciation			
Al ³⁺	9.1	0.1	0.0–9.1
Al(OH) ²⁺	0.3	0.0	0.0–1.3
Al(OH) ₂ ⁺	0.0	0.0	0.0–0.2
AlF ²⁺	6.3	0.6	0.2–6.3
AlF ₂ ⁺	0.2	0.2	0.1–0.6
HCO ₃ [–]	1.3	14	0.7–51
OA [–]	63	53	7–130

Concentrations (in μequivalents l^{–1} except as noted) are simple means of analytical values for samples collected before 1987 drought. Analytical methods and quality control data are described in detail elsewhere^{21–23}. Acid neutralizing capacity was measured by Gran titration, total fluoride (F_T) by ion-selective electrode with TISAB buffer, and organic and inorganic aluminium (Al_{org} and Al_i, respectively) by catechol violet colorimetry before and after ion exchange²⁴. Speciation of aluminium followed the procedure of Schecher and Driscoll²⁵. Organic anions (OA[–]) were calculated from pH and dissolved organic carbon (DOC) by a slight modification¹⁴ of the method of Oliver *et al.*²⁶. Bicarbonate was calculated²⁷ from measured dissolved inorganic carbon (DIC) and pH.

tion was experimentally altered²⁰. Those sites pose a less exacting test, however, because acid loading changed less.

Long-term catchment response to acid loading will be affected by disequilibrium processes, such as base cation leaching, in addition to the equilibrium mechanisms analysed here. Extensions to the theory outlined above yield quantitatively reasonable predictions of the long-term effects of progressive base cation depletion from catchment soils, as may be occurring at Hubbard Brook⁸. Unfortunately, data for a precise test do not exist.

The spatial variability of catchment geochemical properties has little effect on predicted acidification response⁸, because equation (5) is not strongly nonlinear and catchment runoff averages both the solute concentrations and acidification responses of its geochemically diverse contributing regions in similar ways. Consequently, equation (5) is as good a predictor of whole-catchment acidification response at our largest catchment (456 ha) as at our smallest (1 ha). It cannot, however, address the effects of flowpath changes altering the relative flows from geochemically distinct sources, although it will predict acidification response for individual hydrological regimes, from samples taken under those flow conditions (J.W.K., manuscript in preparation).

Equations (5)–(8) are relatively insensitive to the variability of runoff chemistry with time⁸, because they use ratios of concentrations that generally covary in compensating ways. Reliable acidification predictions can usually be obtained from individual runoff samples. Predictions derived from individual streamwater samples differed from the mean predictions at each site by, on average, 0.028 (or 3%) for dSBC/dΣacids, 0.015 (6%) for

dCa/dΣacids, 0.008 (8%) for dMg/dΣacids, 0.026 (18%) for dANC/dΣacids, 0.018 (29%) for dH⁺/dΣacids and 0.005 (64%) for dAl_i/dΣacids. Expected errors in acidification predictions from single samples are therefore much smaller than uncertainties in catchment acidification response before prediction, which for H⁺ and Al_i may exceed an order of magnitude. Therefore, although this critical test uses large data sets from intensively monitored catchments, useful acidification predictions could be derived where only a few samples are available.

Because acid anion changes were largely offset by shifts in base cation concentrations, these acid episodes decreased acid neutralizing capacity only 5–25% as much as they raised acid anion concentrations (Figs 1–3). Catchment base cation and ANC responses to acidification were confined to relatively narrow ranges, as predicted (Figs 2–3). By contrast, acidification affected hydrogen ion and aluminium concentrations at different catchments in markedly different ways, but again, these

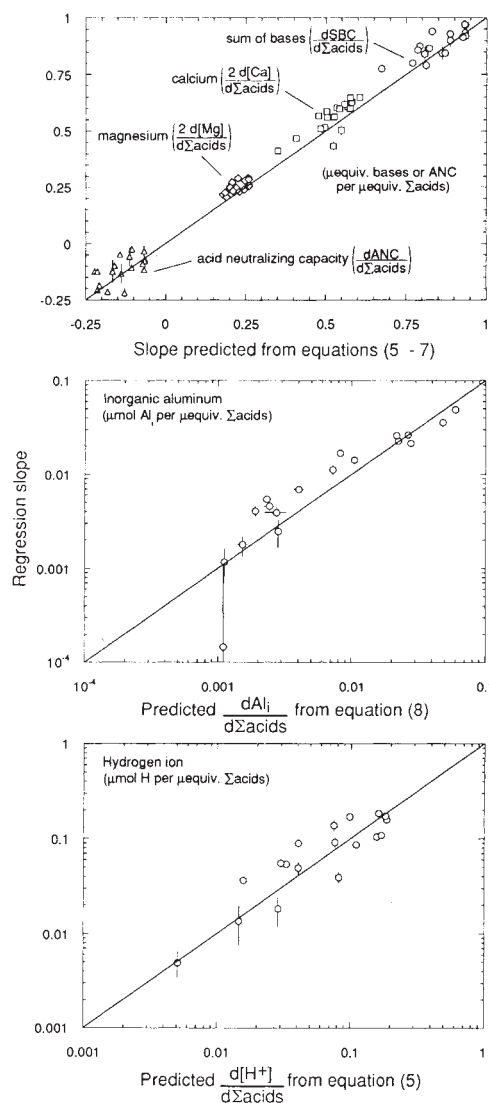


FIG. 3 Comparison of predicted and observed response to shifts in acid anion concentrations in runoff from 18 monitored catchments. Diagonal line indicates perfect agreement. Predicted slopes, like slopes of solid lines in Fig. 2, derived by inserting pre-drought average streamwater concentrations into equations (5)–(8). Observed slopes are least-squares regression slopes of measured concentrations as a function of Σacids, similar to data plotted in Fig. 2. Thus, for each catchment, the experimental data plus predicted line shown in Fig. 2 become a single point here. All samples, both pre-drought and post-drought, are used in calculating observed regression slopes. Error bars show standard errors of prediction and standard errors of regression, where larger than plot symbols.

responses agreed with the theoretical predictions (Figs 2, 3). These results show that runoff chemistry and acidification response are related by simple mechanistic formulas, and that catchment acid sensitivity can be evaluated directly from runoff chemistry data. □

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Patterns in shrinking gels

Eriko Sato Matsuo & Toyochi Tanaka*

Department of Applied Biological Sciences and

*Department of Physics and Center for Materials Science and Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA

POLYMER gels can undergo a volume phase transition (either continuous or discontinuous) when an external condition, such as temperature or solvent composition, is altered^{1–3}. During this transition, the volume may change by a factor of several thousand, and various patterns develop in the gel. The patterns arising from swelling and shrinking differ in both their appearance and their physical mechanisms. The mechanism for the formation and evolution of patterns on swelling gels has been established as being due to a single kind of mechanical instability^{4–7}; in contrast, the shrinking patterns seem to be sensitive to both the initial and final states of the transition. Here we classify the various shrinking patterns in the form of a phase diagram, and explain the polymorphism in terms of macroscopic phase separation.

Cylindrical gels of acrylamide, 0.7 to 10 mm in diameter, were prepared by bulk polymerization in glass tubes of various diameters and dried after being taken out¹. Small amounts of blue dextran ($M_r = 2 \times 10^6$) were added to some gels during

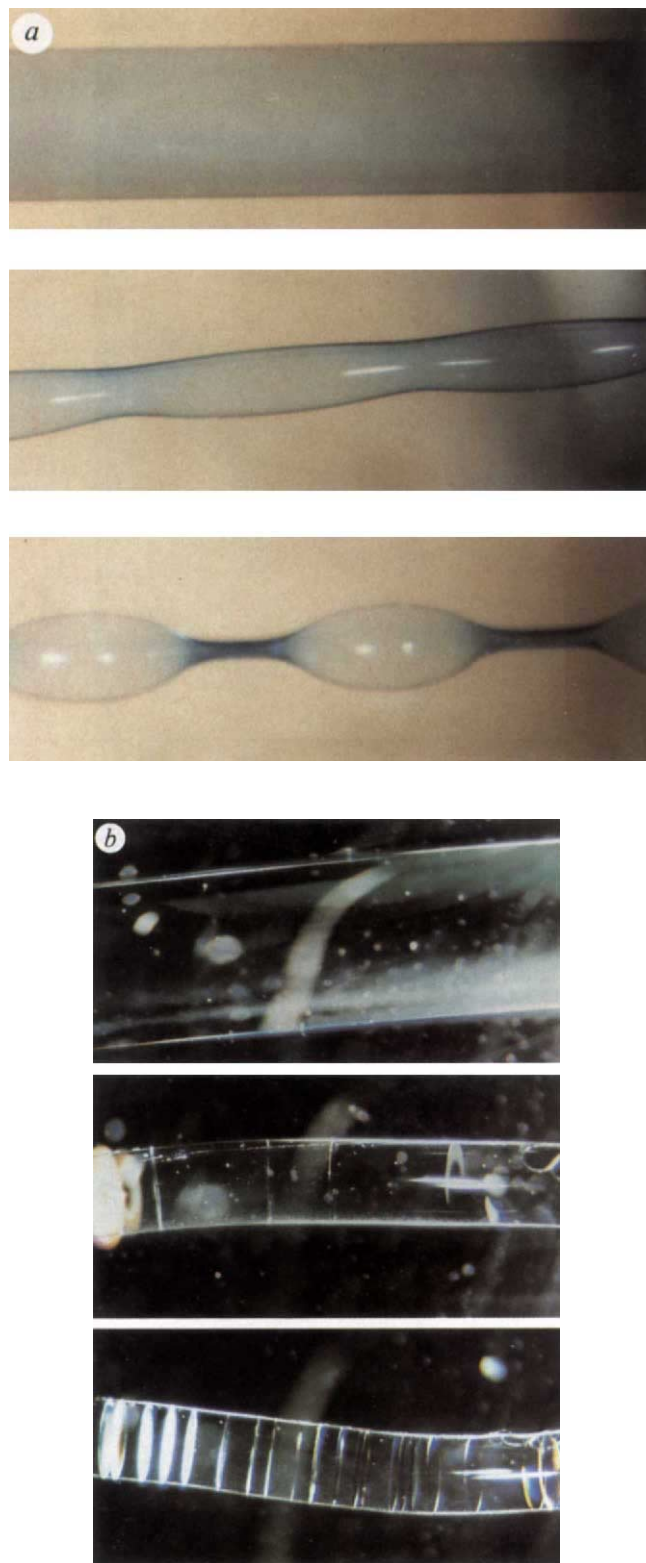


FIG. 1 a, Bubble pattern developing in a shrinking cylindrical acrylamide gel immersed in an acetone–water mixture. The gel is a copolymer of 16 mM sodium acrylate and 700 mM acrylamide. A small amount of blue dextran was added at gelation. The gel length was fixed at 90% of the relaxed length, and placed in 70% (vol%) before mixing) acetone solution. The frames (top to bottom) were taken 0 min, 51 min and 60 min after immersion. b, Bamboo-like pattern in a cylindrical acrylamide gel, consisting of collapsed thin layers perpendicular to the cylinder axis. The number of planes increases with time. The gel is a copolymer of 32 mM sodium acrylate and 700 mM acrylamide. The gel was placed in 85% acetone solution without restricting the length. The times are (top to bottom) 0 min, 14.5 min and 20 min. The magnification is $\times 3.25$ for the top panel and $\times 5$ for the remaining two.

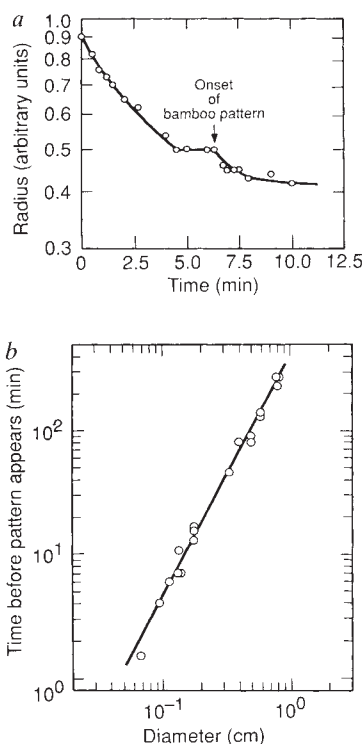


FIG. 2 *a*, Radius of a cylindrical gel as a function of time during the bamboo pattern formation. There is a plateau period during which the radius temporarily stops shrinking. The gel is a copolymer of 16 mM sodium acrylate and 700 mM acrylamide, placed in 85% acetone solution without fixing the length. *b*, Length of time before onset of bamboo formation is proportional to the square of gel diameter. The gel is a copolymer of 8 mM sodium acrylate and 700 mM acrylamide, placed in 95% acetone solution without fixing the length.

polymerization to aid observation. Each end of the dried gel was glued to a plastic tip fixed on a glass rod; the separation of the tips was varied to control the final length of the gel. The gel was allowed to swell in water and, after it reached equilibrium, placed in an acetone-water mixture.

We investigated the dependence of the patterns on the acetone composition and final length of the gel. Under certain conditions, a 'bubble' pattern was observed (Fig. 1*a*). The gel at first shrank keeping its smooth shape until it reached a certain radius. At the end of a plateau period during which the radius remained constant, alternate swollen and shrunken portions appeared. Similar patterns and behaviour have been reported for spherical gels⁸.

In other cases, 'bamboo' patterns were observed (the different conditions required are described in Figs 1 and 3). Again, at the end of a plateau period cross-sectional planes consisting of the collapsed gel membrane appeared in the cylinder. The planes had a thickness comparable to the wavelength of light, as shown by the rainbow colours of the reflected light (Fig. 1*b*). After the spatial frequency reached a certain value, the total gel radius decreased. The sequence of radial change of a cylindrical gel developing the bamboo pattern is shown in Fig. 2*a*. The onset of pattern formation and the plateau period are clearly seen. The time taken for the pattern to appear was proportional to the square of gel radius (Fig. 2*b*). The same rule applied to the bubble pattern⁹.

When the gel was immersed in a highly concentrated acetone solution (near 100%), it became opaque and wrinkled. The gel eventually became hollow, forming a tube. Evidently the polymer network had disappeared.

Figure 3 shows a 'phase diagram' representing all the observed patterns for various combinations of acetone concentration and final fixed length of the gel. We also did some preliminary work on the effect of ionization of gels, making the ionized gels by copolymerization of acrylamide and sodium acrylate. The effect was to shift the phase diagram towards higher acetone

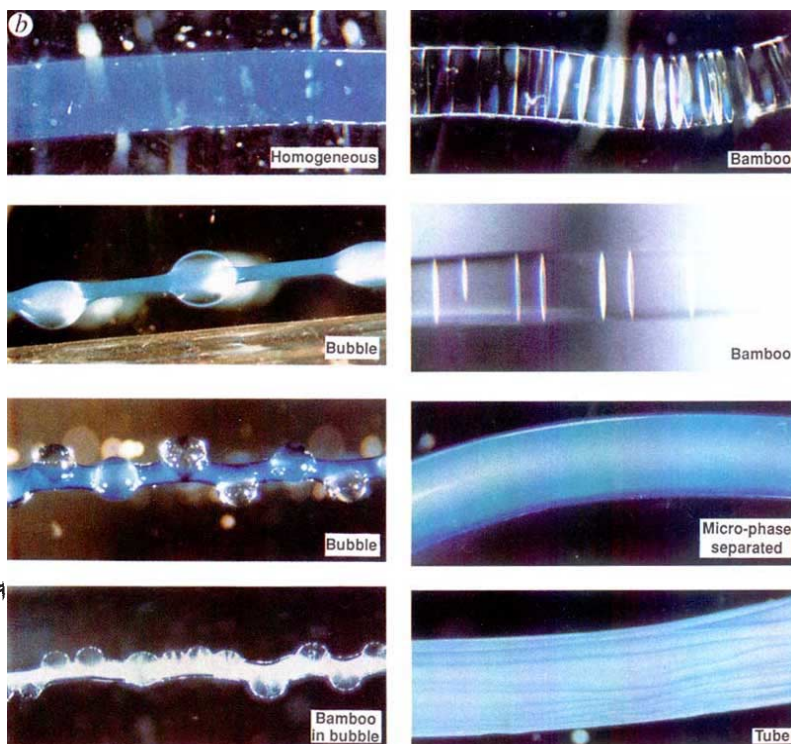
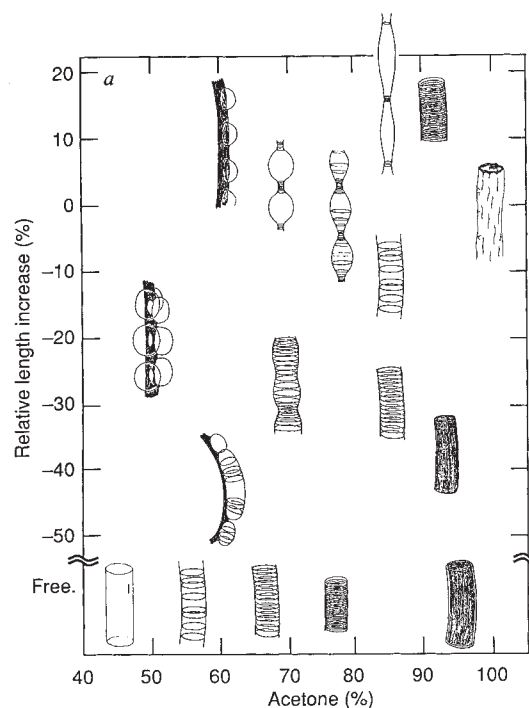


FIG. 3 *a*, 'Phase diagram' showing the regions of acetone concentration and fixed final length of a gel at which different patterns of cylindrical acrylamide gels are formed. A negative relative length increase indicates that the fixed

length was shorter than the original relaxed length, whereas a positive value means that the gel was stretched before shrinking. *b*, Photographs that best represent the various patterns. Some are of ionic gels.

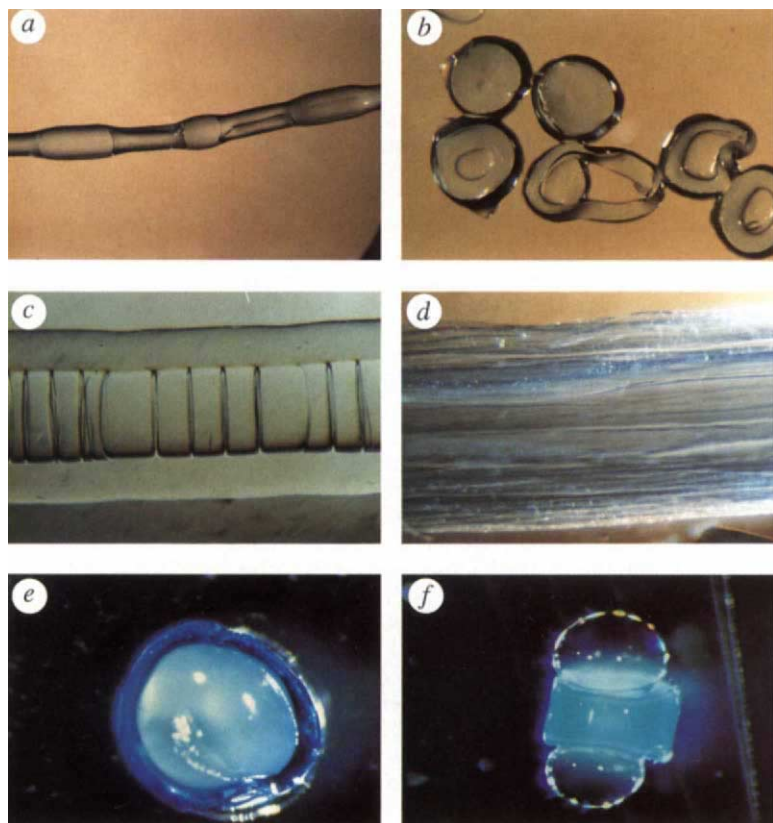


FIG. 4 Reswollen gels show permanent alterations in the gel structure after formation of patterns. *a*, Whole cylinder of a bubble, *b*, its cross-sections. The bamboo (*c*) and wrinkled tube (*d*) were cut along their cylindrical axes. Cross-sections of a gel at the early stage of the bubble pattern formation are also shown. First, the gel was cut in air (*e*). We can clearly see the formation of a dense impermeable layer. The opaque core shows the presence of microscopic domains at the early stage of phase separation. A build-up of internal pressure is indicated by the bubble formation at the newly exposed surface of a gel cut in an acetone-rich solution (*f*).

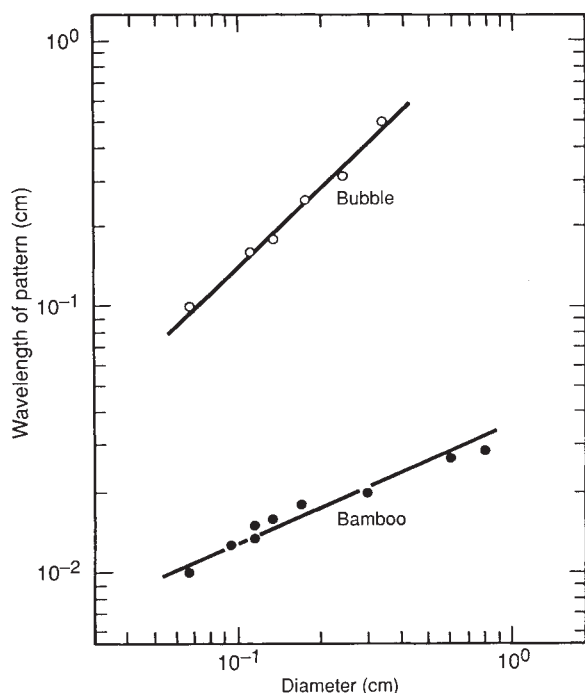


FIG. 5 Wavelengths of bamboo and bubble (pearl-necklace) patterns as a function of gel radius. The bamboo pattern is from a gel of sodium acrylate (8 mM)/acrylamide (700 mM) in 90% acetone solution; the bubble pattern is from a gel of 700 mM acrylamide in 60% acetone. A small amount of blue dextran was added to the latter gel. The power slopes were 0.5 for bamboo and 1 for bubble.

concentration and to widen the region in which a combined bamboo and bubble pattern⁹ was seen. Once formed, the bamboo, tube and some bubble patterns were very stable and did not disappear for months or even for years. The dilute region of the polymer network in all the patterns became hollow at some point. Apparently the polymer chains were broken. This was confirmed by allowing the gels with patterns to swell again in water as shown in Fig. 4. Hollow structures have also been seen to develop in polymer fibres^{9,10}.

Pattern formation in shrinking gels seems to be explained by the formation of a dense impermeable layer, build-up of internal pressure and phase separation under fixed volume. The three processes can be seen in the cross-sections of a cylinder gel leading to bubble formation as shown in Fig. 4.

Because a gel shrinks by the cooperative diffusion of the polymer network, the shrinkage starts from the gel boundary^{11,12}. In the initial stages, the density is high only at the gel surface. The interior of the gel still has the original low density. If the density of the collapsed layer remains fairly low and permeable to water, the gel continues to shrink into a homogeneous shape. If the layer becomes very dense and impermeable to water, the gel volume is fixed and there is no more shrinkage. This is the onset of the plateau period. At this time the polymer density and osmotic stress are not homogeneous within the gel. They relax under the constant-volume condition imposed by the impermeable layer.

After the relaxation the inner portion of the gel is still swollen, although the solvent is extremely poor and the reduced temperature (free energy of polymer segments in contact divided by kT) is very negative. The state of the gel may be below the spinodal line (supercooled), and the gel undergoes a spontaneous phase separation into domains of collapsed and swollen phases. Under the constant-volume condition, some regions shrink at the cost of the other regions, which are forced to expand. If the outer layer is flexible, the boundary changes its shape and the bubble pattern is formed. If the stretched layer

becomes permeable again, the gel resumes shrinking and reaches a homogeneous state; if it remains impermeable, a permanent pattern is formed. In contrast, if the skin is not flexible and the boundary is fixed, the phase separation produces a wave along the axis (bamboo), or in the radial direction (tube). If the reduced temperature is very low and the driving force for phase separation is strong, polymers within swollen domains may be ruptured, allowing the shrunken portion of the polymer network to collapse completely. The wavelength of the patterns is proportional to the square of gel radius for the bamboo pattern and to radius for the bubble pattern (Fig. 5).

We believe that the work presented here will be relevant to the formation and evolution of patterns found in the biological realm. □

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Changes in surface salinity of the North Atlantic Ocean during the last deglaciation

J. C. Duplessy*, L. Labeyrie*, M. Arnold*, M. Paterne*, J. Duprat† & T. C. E. van Weering‡

* Centre des Faibles Radioactivités, Laboratoire mixte CNRS-CEA, 91198 Gif sur Yvette Cedex, France

† Laboratoire de Géologie et Océanographie, Université de Bordeaux 1, 33405 Talence, France

‡ Nederlands Instituut voor Onderzoek der Zee, Postbox 59, 1790 AB den Burg, Texel, The Netherlands

ABRUPT and short climate changes, such as the Younger Dryas, punctuated the last glacial-to-interglacial transition^{1–4}. Broecker *et al.*⁵ proposed that these may have been caused by an interruption of thermohaline circulation as inputs of glacial meltwater freshened the surface waters of the North Atlantic. The finding⁶ that meltwater discharge was minimal during the Younger Dryas, however, led to the suggestion that the surface-water salinity drop might have been caused instead by changes in the freshwater budget (the difference between precipitation and evaporation), accompanied by a reduction in poleward advection of saline subtropical water. Here we use micropalaeontological and stable-isotope records from foraminifera in two cores from the North Atlantic to generate two continuous, high-resolution records of sea surface temperature and salinity changes over the past 18,000 years. Despite the injection of glacial meltwater during warm episodes, we find that sea surface salinity and temperature remain positively correlated during deglaciation. Cold, low-salinity events occurred during the early stages of deglaciation (14,500–13,000 years ago) and the Younger Dryas, but the minor injections of meltwater at high latitudes during these events are insufficient to account for the observed salinity changes. We conclude that an additional feedback from changes in the hydrological cycle and in advection was necessary to trigger changes in thermohaline circulation and

TABLE 1 Key AMS radiocarbon dates in core NA 87-22

Depth (cm)	Conventional radiocarbon age (yr)	Standard deviation (yr)	Foraminifer
95	3,520	90	<i>N. pachyderma</i> (right)
200	8,280	110	<i>G. bulloides</i>
250	10,010	90	<i>G. bulloides</i>
270	10,870	140	<i>G. bulloides</i>
285	11,320	90	<i>N. pachyderma</i> (left)
305	11,690	120	<i>G. bulloides</i>
325	12,380	120	<i>G. bulloides</i>
355	15,660	120	<i>N. pachyderma</i> (left)
380	16,700	140	<i>N. pachyderma</i> (left)
400	17,590	140	<i>N. pachyderma</i> (left)
440	19,310	270	<i>N. pachyderma</i> (left)

thus in climate. This feedback did not act when the meltwater injection occurred at low latitude.

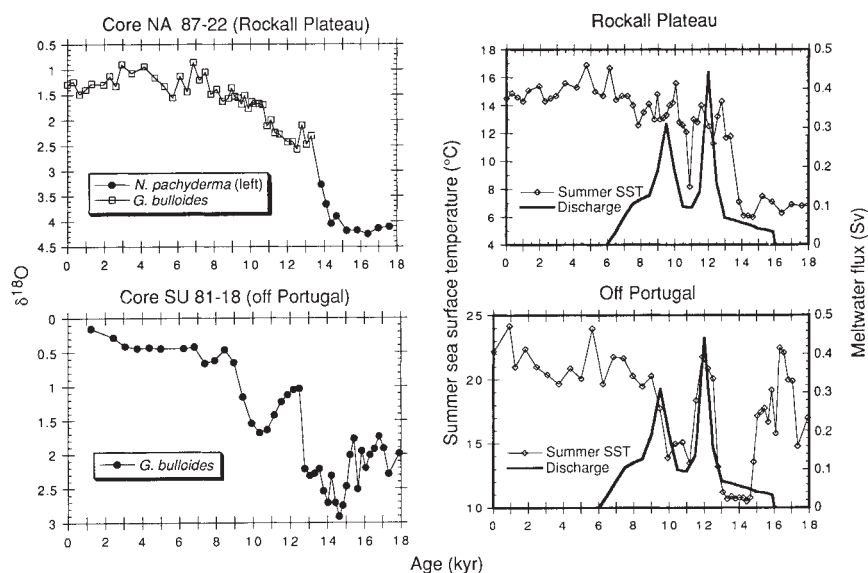
One of the cores discussed here was recovered off the Rockall Plateau (NA 87-22; 55° 30' N, 14° 42' W, 2,161 m water depth), and the second is located off Portugal (SU 81-18; 37° 46' N, 10° 11' W, 3,135 m water depth). Radiocarbon measurements of monospecific foraminiferal samples from core SU 81-18 have been reported previously⁷. Those for core NA 87-22 are reported in Table 1. Only peaks of abundance of *Globigerina bulloides* and *Neogloboquadrina pachyderma* (left-coiling) were dated. The $\delta^{18}\text{O}$ input curve has been constructed from the sea-level record from Barbados⁸. In addition to the original ^{14}C data, we used analyses obtained by accelerator mass spectrometry (AMS) at Gif⁹. To work on a common timescale for the two sediment cores and the Barbados coral record, we corrected the conventional ^{14}C ages for the reservoir age (400 years) of surface water^{9,10}. Finally, we obtained the timescale for the two deep-sea cores by fitting a fifth-order polynomial curve to the profiles of ^{14}C age against depth.

We consider two palaeoclimate signals in the deep-sea sediment record (Fig. 1). Sea surface temperature (SST) estimates were based on micropalaeontological transfer functions^{11,12}, and oxygen isotope measurements ($\delta^{18}\text{O}$) were made on the planktonic foraminifera *G. bulloides* in core SU 81-18, and on *G. bulloides* and *N. pachyderma* (left-coiling) in core NA 87-22. Both species were selected because they accurately record sea surface $\delta^{18}\text{O}$ and temperature variations as long as the summer SST remains within a well-defined temperature range, characteristic of each species and referred to as the 'optimal temperature range'¹³. But the temperature thus recorded is neither the summer nor the mean annual temperature, because foraminifera live only during part of the spring and summer blooms. Statistical analysis of the $\delta^{18}\text{O}$ values of modern samples of *G. bulloides* and *N. pachyderma* (left), and the temperature of the waters from which they were collected, showed that the isotopic temperatures T^* indicated by these species are summer SST -1°C for *G. bulloides*, and summer SST -2.5°C for *N. pachyderma* (left)¹³.

Surface water $\delta^{18}\text{O}$ variations at the location of the two cores were calculated from the foraminiferal $\delta^{18}\text{O}$ records and the SST estimates by solving the palaeotemperature equation^{14,15} and assuming that the planktonic foraminifera deposited their shells in isotopic equilibrium with the growth temperature (T^*). These $\delta^{18}\text{O}$ variations reflect salinity changes resulting from three causes, namely continental ice melting, changes in the balance of precipitation and evaporation ($P - E$), and changes in rate of advection and mixing within the Atlantic Ocean^{16–18}, whose effects are additive.

We calculated first the surface water $\delta^{18}\text{O}$ variations due to ice-sheet melting. Some geographical $\delta^{18}\text{O}$ differences are to be expected, because the meltwater was introduced first into the North Atlantic Ocean and was progressively diluted in the other ocean basins. We calculated the temporal changes of North

FIG. 1 Stable isotope records and sea surface temperature estimates derived from cores NA 87-22 (Rockall Plateau, off Ireland³⁶) and SU 81-18 (off Portugal). The flux of meltwater discharge due to the melting of continental ice sheets was obtained by calculating the derivative of the Barbados sea-level curve (ref. 6). $\delta^{18}\text{O} = \{(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}}\} - 1$, where standard is PDB.



Atlantic surface water $\delta^{18}\text{O}$ using a four-box model of oceanic circulation^{19,20}. We calculated the dilution of the meltwater input assuming that the flux of North Atlantic Deep Water (NADW), which determines the residence time of surface Atlantic water in the model, was the same during the last deglaciation as it is today. As the ice that melted during the early phase of the last deglaciation was less depleted in ^{18}O than during the end of the climate transition²¹, we assumed that the $\delta^{18}\text{O}$ of meltwater varied linearly with ice volume from -20% 16,000 years ago to -40% at the end of the deglaciation 6,000 years ago. None of the following conclusions is dependent on this hypothesis, which shows only minor differences from melting of an ice sheet with an homogenous isotopic composition²². The resulting salinity change was estimated assuming that salinity increased by 1 when the seawater $\delta^{18}\text{O}$ increased by 1.2‰ (the mean value of three independent estimates^{6,23,24}).

We define a local $\delta^{18}\text{O}$ anomaly, which is due to changes in $P-E$ and advection, as the difference between the measured foraminiferal $\delta^{18}\text{O}$ value and that owing to input of meltwater. The corresponding local salinity anomaly was estimated assuming that salinity increased by 2 when seawater $\delta^{18}\text{O}$ increased by 1‰ (ref. 25). Details of the calculations for core SU 81-18 have been given previously²². Data for core NA 87-22 will be published elsewhere (L.L. *et al.*, manuscript in preparation).

The SST records of core SU 81-18 and NA 87-22 are compared with the meltwater input in Fig. 1. For both cores, the Younger Dryas cold event is well marked. Low SST, recorded in core NA 87-22, prevailed off Ireland until $\sim 13,500$ yr before present (BP). At the lower latitude location of core SU 81-18, warm SST prevailed during the last glacial maximum because the steep thermal gradient that separated the subtropical waters from the polar water mass was located north of 38°N (ref. 26). A sharp cooling ($\sim 8^\circ\text{C}$) occurred between 14,500 and 13,000 yr BP off Portugal and coincides with the meltwater discharge believed to have come from the Barents and the Fennoscandian ice-sheets^{27,28}. This event most probably coincides with the Oldest Dryas event recognized in northwestern Europe²⁹ and is marked by low SST in both our cores. By contrast, the main peaks of meltwater discharge coincide with warm SST; the first occurs during the warm Bolling/Allerod interval, whereas the second is associated with the postglacial warming.

In Fig. 2 we compare the estimated sea surface salinity anomaly in cores NA 87-22 and SU 81-18 with the meltwater discharge. For core NA 87-22, the salinity anomaly is not significantly different from zero during the past 9,000 years and during the Bolling/Allerod interval. In particular, no significant

additional salinity decrease is observed during the peaks of meltwater discharge, which coincide with warm periods. In core SU 81-18, the presence of subtropical water produces a positive salinity anomaly during the Bolling/Allerod interval, reflecting the strong evaporation which affects the subtropics. During the last glacial maximum, about 18,000 years ago, the anomaly was negative at the location of core NA 87-22 and close to zero for core SU 81-18, which was located in the warm water²⁶.

In both cores, sharp negative salinity anomalies are associated with the Younger Dryas and the Oldest Dryas cold events. This indicates that the surface salinities of the high latitudes of the North Atlantic Ocean were lower than those calculated by assuming that fluxes of NADW and $E-P$ were similar to the

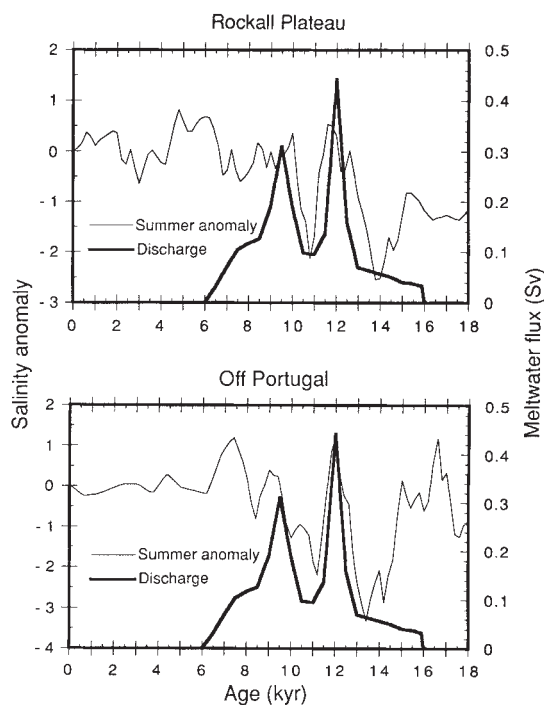
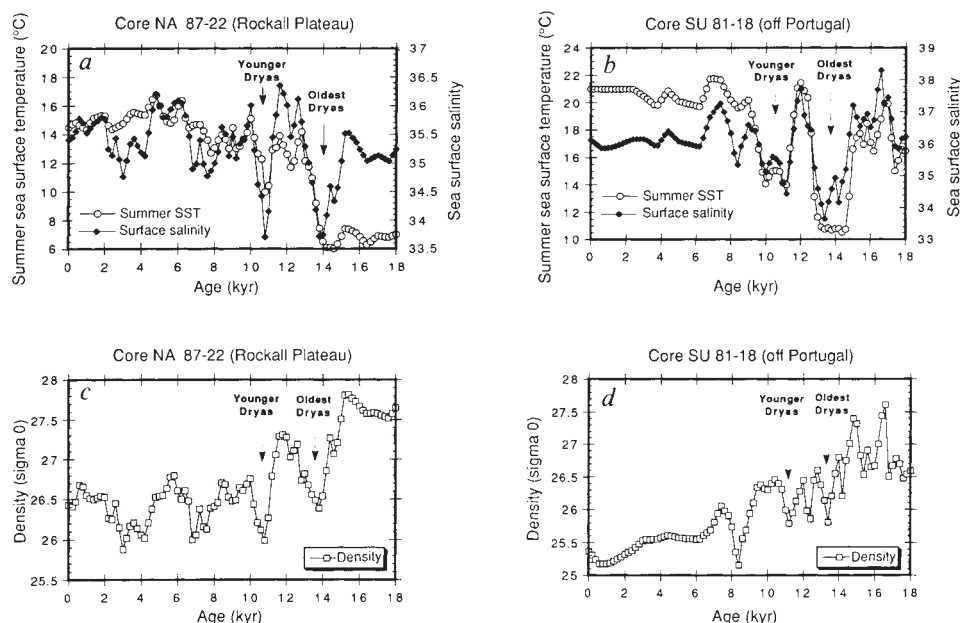


FIG. 2 Estimates of sea surface salinity anomalies derived from the isotope and micropalaeontological analyses of cores NA 87-22 (Rockall Plateau, off Ireland) and SU 81-18 (off Portugal). The flux of meltwater discharge due to the melting of continental ice sheets was obtained by calculating the derivative of the Barbados sea-level curve (ref. 6).

FIG. 3 Sea surface temperature and salinity estimates for cores NA 87-22 (a), SU 81-18 (b) and the associated surface water density changes (c, d). All values have been interpolated every 200 yr. Densities are expressed as sigma 0 = $(\rho - 1) \times 1,000$.



present. Such negative salinity anomalies result either from a considerable reduction in the strength of the thermohaline 'conveyor belt'^{30,31} accompanied by small changes in the hydrological cycle, or from a strong freshwater injection owing to changes in the local $P - E$ budget. A common characteristic of both events is the injection of significant amounts of freshwater in the high latitudes of the North Atlantic^{5,27,28}, with fluxes varying from 0.05 to 0.1 Sv (Fig. 2). These fluxes, which correspond to a zero salinity anomaly in our computation scheme, are not sufficient by themselves to produce the low surface salinity observed in the North Atlantic Ocean (Fig. 2). Model experiments showed, however, that they can strongly reduce the formation of NADW and the Atlantic meridional heat transport^{16,32}. Our measurements imply that the combined role of reduced advection of high salinity surface water and changes in the hydrological cycle resulted in an additional salinity drop. This positive feedback was so fast that both the SST and salinity drops appear to be in phase with the freshwater input associated with the melting of the Barents ice sheet during the Oldest Dryas^{27,28}, and the diversion of the meltwater discharge from the Mississippi to the Saint Lawrence rivers during the Younger Dryas⁵. By contrast, our measurements show that an injection of freshwater four times larger in the Gulf of Mexico at low latitude does not disrupt the North Atlantic thermohaline circulation and is not amplified by the hydrological cycle. The absence of feedback to the meltwater injection results in only a moderate salinity change, with no significant temperature drop at high latitude and therefore no major reduction of the intensity of the Conveyor Belt. This was noticeably the case during the warm Bolling/Allerod events.

Sea surface salinity estimates at the location of cores SU 81-18 and NA 87-22 have been obtained by adding to the modern salinity value the changes due to meltwater and to advection and $P - E$ changes. These estimates together with those of SST and density³³ are shown in Fig. 3. The main salinity drops associated with the Younger and Oldest Dryas events resulted in density drops larger than 0.5‰. At these times, meltwater discharge was small⁶ and the deep-water density did not change greatly³⁴. The surface density decrease therefore greatly enhanced the stratification of the North Atlantic and reduced the production of NADW.

Our data show that large variations in the salinity of the northern North Atlantic occurred during the past 18,000 years, which were linked to changes in the strength of thermohaline

circulation^{16,17,35}. A primary forcing factor for surface salinity was the injection of meltwater in the northern North Atlantic, which led to increasing water stratification and decreasing SST. Both effects led to decreased evaporation, increased precipitation and reduced poleward advection of salty subtropical water. These feedbacks amplified the initial freshwater signal, resulting in a shutdown or a weaker flux of NADW, a reduced heat transport to high latitudes and cold conditions over the northern North Atlantic and the neighbouring European continent. By contrast, in disagreement with model simulations^{16,32}, our data show that SST and salinity in the northern North Atlantic were not affected when the freshwater input occurred at low latitude. Here the amplifying feedbacks did not operate, so that the principal phases of ice-sheet melting were associated with warm climate conditions over the North Atlantic and active thermohaline circulation favouring meltwater dilution in the world ocean¹³. □

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Post-rifting stress relaxation at the divergent plate boundary in Northeast Iceland

G. R. Foulger*, C.-H. Jahn†, G. Seeber†, P. Einarsson‡, B. R. Julian§ & K. Heki*||

*Department of Geological Sciences, University of Durham, Science Laboratories, South Road, Durham DH1 3LE, UK

†Institut für Erdmessung, Universität Hannover, D-3000 Hannover 1, Neiburger Strasse 5, Germany

‡Science Institute, University of Iceland, Dunhaga 3, Reykjavik, Iceland

§U.S. Geological Survey, 345 Middlefield Road, MS 977, Menlo Park, California 94025, USA

INTERACTION of the elastic lithosphere with the underlying anelastic asthenosphere causes strain to propagate along the Earth's surface in a diffusion-like manner following tectonism at plate boundaries. This process transfers stress between adjacent tectonic segments and influences the temporal tectonic pattern along a plate boundary. Observations of such strain transients have been rare, and have hitherto been confined to strike-slip and underthrusting plate boundaries¹. Here we report the observation of a strain transient at the divergent (spreading) plate boundary in Iceland. A Global Positioning System survey undertaken a decade after an episode of dyke intrusion accompanying several metres of crustal spreading reveals a spatially varying strain field with the expected diffusion-pulse shape and an amplitude three times greater than the 5.7 cm that would be expected from the average spreading rate². A simple one-dimensional model with a thin elastic layer overlying a viscous layer fits the data well and yields a stress diffusivity of $1.1 \pm 0.3 \text{ m}^2 \text{ s}^{-1}$. Combined with structural information from magnetotelluric measurements, this implies a viscosity of $0.3\text{--}2 \times 10^{19} \text{ Pa s}$ —a value comparable to that derived for Iceland from post-glacial rebound²³, but low compared with estimates for mantle viscosity obtained elsewhere³.

The Krafla volcanic system is one of five *en echelon* systems in the Northern Volcanic Zone of Iceland, the locus of the accretionary plate boundary there⁴ (Fig. 1a). It comprises a 100-km-long fissure swarm containing the Krafla central volcano. The full spreading rate predicted by the NUVEL plate velocity model is 1.9 cm yr^{-1} (ref. 2).

A spreading episode commenced in the Krafla system in 1975 (refs 5–10). During the following decade a magma chamber beneath the volcano inflated, with occasional rapid deflations when magma flowed out of the chamber and was intruded along the fissure swarm, forming dykes. From 1975 to 1979, about 10 such deflations with dyke intrusion occurred. Terrestrial surveying work revealed that roughly a metre of local crustal widening accompanied each event. The total widening across the fissure swarm was ~2 m along 50 km of its length, and 6 m along another 30 km, giving an average expansion of 3.5 m (ref. 10). The average height of the dyke complex was estimated to be 2 km, assuming that its width was constant with depth¹⁰. The

widening zone extended from Axarfjörður in the north to Hverfjall in the south (Fig. 1a). Expansion in the centre of the fissure swarm was accompanied by contraction of the adjacent plates out to at least 50 km from the fissure swarm^{11,12}.

After 1980, most of the magma escaping during deflations flowed onto the surface as lava. Evidently the stress perpendicular to the plate boundary had increased so as to prevent further dyke intrusions. Post-intrusion earthquake focal mechanisms show the absence of a systematic extensional stress field, thereby supporting this conclusion^{13,14}.

Early geodetic studies indicate that little spreading occurred in the Krafla system during the 27-yr period 1938–65 (ref. 15). Historical records, however, describe eruptions and tectonism during the period 1724–29 that were similar to those of 1975–85. These observations suggest that crustal spreading is episodic in the Krafla system, with several metres of widening occurring during brief episodes of intense activity, separated by quiescent periods lasting hundreds of years.

A geodetic network of ~40 points encompassing Northeast Iceland was measured using the Global Positioning System

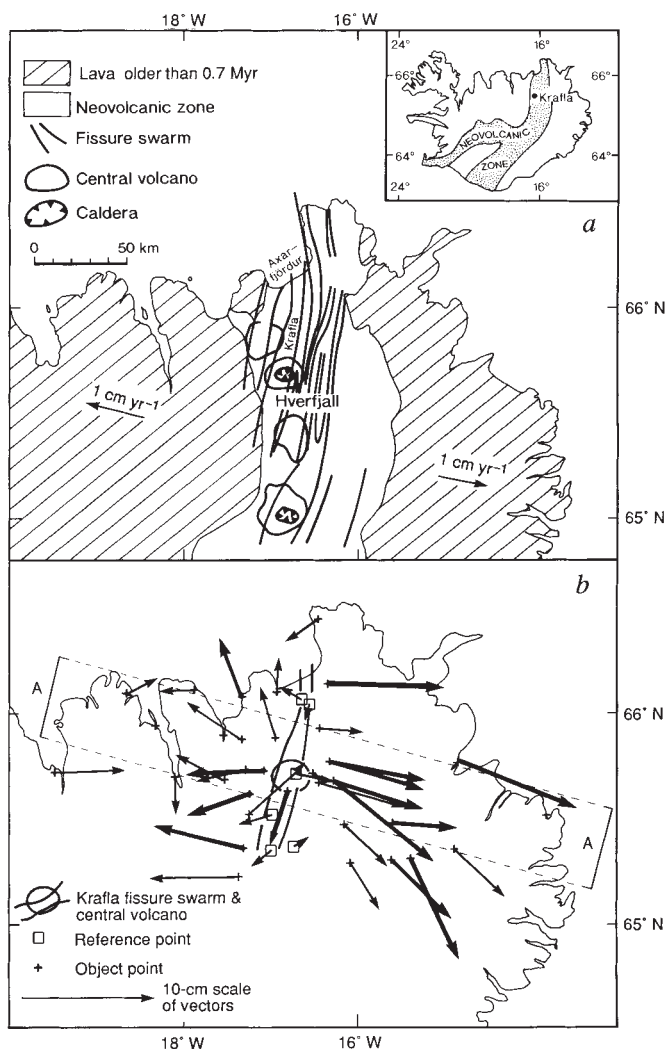


FIG. 1 a, Tectonic map of Northeast Iceland showing the five *en echelon* volcanic systems that comprise the accretionary plate boundary. b, Horizontal point displacements, 1987–90. The lengths of the vectors are proportional to the point displacements. Thick lines indicate vectors that are significant at greater than the 99% level (3σ). Zone A indicates the part of the network around the middle of the intruded dyke complex, and points within this zone are modelled in Fig. 2. Zone A extends from 26.5 km north of the point on Mount Krafla to 16.9 km south of it, parallel to the trend of the volcanic system.

(GPS) in 1987 and remeasured in 1990 (Fig. 1b). Points were concentrated within the Krafla volcanic system and were sparsely distributed for 130 km into the adjoining plates. In 1987 a six-satellite daily observation window 3 hr 20 min long was used, and most of the points were occupied two or three times. The data were processed at the University of Hannover with GEONAP (geodetic navstar positioning) software¹⁶ and at the University of Durham with the Bernese version 3.2 GPS software¹⁷. The standard deviations of all the components are ~ 0.5 – 1.3 cm for the GEONAP results^{15,18}, and the integer values of all cycle slips and ambiguities could be calculated. The GEONAP and Bernese results agree to within 1 cm in the horizontal for more than 70% of the points. Broadcast ephemerides were used for both analyses, as the available precise orbits offered little improvement.

In 1990, a seven-satellite daily observation window of 4 hr 10 min was used². Selective Availability (degradation of signal quality for military security) was on for most of the survey. The 1990 data are noisy because of ionospheric irregularities (scintillations) caused by the current sunspot maximum, but again, the integer values of cycle slips and ambiguities could be calculated using suitable linear combinations of the carrier-phase data. The 1990 GEONAP and Bernese solutions yielded standard deviations (1σ) of 1–3 cm for all components after network adjustment. The agreement between the GEONAP¹⁹ and Bernese results is mostly within the 1σ error. We assumed a standard troposphere in all our analyses, an adequate model in the low-temperature environment of Iceland. Because of the great ionospheric turbulence, the 1990 results¹⁸ are inferior to those from 1987.

The deformation results described here are based on the GEONAP results. The two coordinate sets with their complete variance-covariance matrices were subjected to deformation analysis using the PANDA¹⁹ software. This corrected for systematic translations and rotations between epochs arising from unmodelled systematic errors using a set of reference points close to the rift axis that showed no significant scale deformation (Fig. 1b). The displacement field shows the relative motion of the other points (object points) with respect to the reference points. Significant movements occurred for 44% of the object points. The deformation field shows a large, systematic east-west expansion with a maximum amplitude of ~ 15 cm. This is clearest around the middle of the fissure swarm, where crustal widening was greatest during 1975–85 (zone A, Fig. 1b). Vertical movements were up to 10–15 cm, but no systematic deformation pattern such as occurs in the horizontal plane was observed.

The time-averaged spreading rate in Northeast Iceland,

1.9 cm yr^{-1} (ref. 2), cannot account for the deformation. Also, no significant tectonic activity occurred in our network between 1987 and 1990. We therefore attribute the 1987–1990 deformation to consequences of the 1975–85 spreading episode. We show below that the 1987–90 deformation agrees well with the field predicted for such an episode by the simple model of transient stress relaxation proposed originally by Elsasser²⁰ and Bott and Dean²¹.

The Elsasser–Bott–Dean model consists of a thin elastic layer overlying a viscous layer (Fig. 2a). We will consider the deformation source as a long vertical dyke oriented in the y -direction, with x being the horizontal distance from the dyke and t being time. This problem is one-dimensional with the only non-zero component of horizontal displacement, $u(x, t)$, lying in the x -direction. If the traction exerted by the viscous layer on the base of the elastic layer is $-(\eta/b) \partial u / \partial t$, where η and b are the viscosity and thickness of the viscous layer, then u obeys the diffusion equation^{20,21}

$$\frac{\partial u}{\partial t} = \kappa \frac{\partial^2 u}{\partial x^2} \quad (1)$$

with

$$\kappa = Mhb/\eta \quad (2)$$

where h is the thickness of the elastic layer and M is the elastic modulus relating horizontal stress and strain within it. For the plane-strain conditions assumed here (in the x – z plane), $M = 4\mu(\lambda + \mu)/(\lambda + 2\mu)$, where λ and μ are the Lamé moduli. The elastic layer is initially at rest, and at time $t = 0$ a dyke of width $2U_0$ is intruded, so that the material immediately adjacent to the line $x = 0$ is suddenly displaced to the right or left by U_0 . The appropriate solution to the diffusion equation (1) is²²

$$u(x, t) = U_0 \operatorname{erfc} \frac{x}{2\sqrt{\kappa t}} \quad (3)$$

and the corresponding horizontal velocity is

$$\frac{\partial u}{\partial t} = \frac{U_0}{t\sqrt{\pi}} \frac{x}{2\sqrt{\kappa t}} e^{-x^2/4\kappa t} \quad (4)$$

We treat the observed 3-yr displacements as instantaneous velocity 11 years after the dyke intrusion episode, and estimate the half-width of the dyke, U_0 , and the diffusivity, κ , by fitting the theoretical expression for the velocity, equation (4), to the observations.

Figure 2b illustrates the best-fit curve, in the least-squares sense, to the point displacements around the middle of the dyke

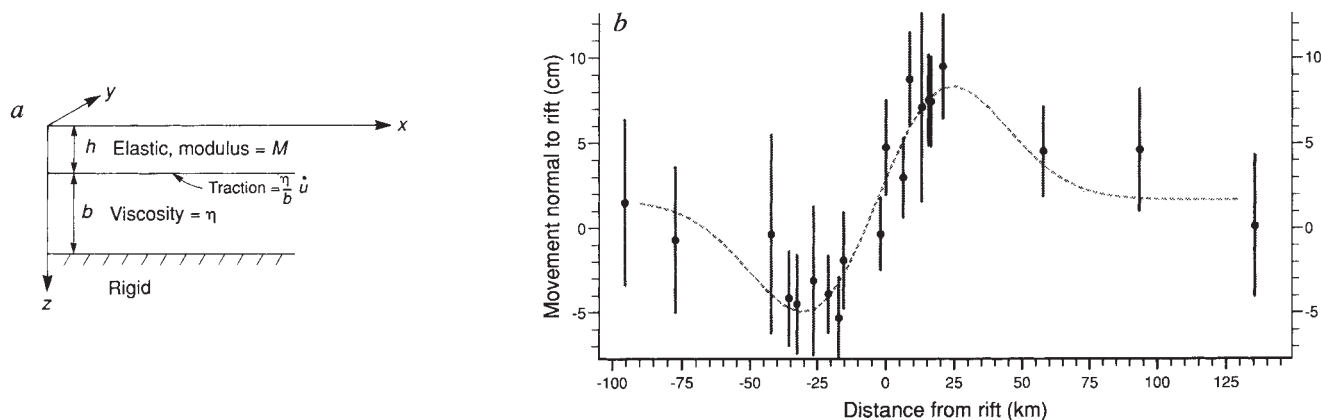


FIG. 2 a, Schematic diagram of the Elsasser–Bott–Dean model. b, Movements of points within zone A (Fig. 1b), perpendicular to the fissure swarm as a function of distance from it. The trend of the plate boundary is taken to be 015° . Vertical bars indicate 1σ error bars. The curve superimposed on the data points is the best-fit curve, in a least-squares sense, of the form

predicted by the diffusion equation. It corresponds to a dyke half-width of 1.0 ± 0.1 m and a diffusivity of $1.1 \pm 0.3 \text{ m}^2 \text{ s}^{-1}$. This model has a normalized root-mean-square residual of 0.61 and a weighted root-mean-square residual of 1.68 cm.

complex, zone A (Fig. 1b). The position of the centre of the dyke is well known from previous geodetic measurements and was held to a fixed location within the fissure swarm. The analysis indicates a dyke half-width of 1.0 ± 0.1 m and a diffusivity of $1.1 \pm 0.3 \text{ m}^2 \text{ s}^{-1}$. This estimate of U_0 suggests a value of 2.0 m for the average width of the dyke complex, which is smaller than the 3.5 m obtained from geodetic measurements during the spreading episode, as expected if the dykes extend only part of the way through the elastic layer. The distance of the velocity maximum from the dyke, and therefore the value of the diffusivity obtained, $1.1 \text{ m}^2 \text{ s}^{-1}$, is fairly well constrained.

Equation (2) may be used to estimate the value of b , h or η , provided the two other parameters are assumed. Magnetotelluric work suggests that Northeast Iceland is underlain by a layer of partial melt 5–10 km thick, and it has been suggested that this layer decouples an overlying layer from material with a high viscosity below¹⁰. If we identify this partial melt layer with the viscous layer in our model, and take $h = 8$ –30 km, $b = 5$ –10 km, $\lambda = \mu = 0.28 \times 10^{11}$ Pa for basalt and $\kappa = 1.1 \text{ m}^2 \text{ s}^{-1}$, we calculate $\eta = 0.3$ – 2×10^{19} Pa s. This may be compared with values of 10^{19} Pa s obtained for the viscosity in a half-space model of Icelandic post-glacial rebound²³.

The rebound estimate of viscosity is not significantly different from ours. Our estimate of diffusivity, however, is incompatible with a thick layer having a viscosity as low as we have calculated. These two apparently incompatible results can be reconciled by a model that involves a thin, shallow layer of relatively low viscosity ($\ll 10^{19}$) underlain by a thicker layer of higher viscosity ($\sim 10^{19}$). The thin, shallow layer may lubricate horizontal motions in the manner suggested by Björnsson¹⁰ and at the same time transmit stress from the large-scale glacial relief to deeper regions.

Our model is a simplification, and although the data have the essential shape predicted by diffusion theory, our quantitative interpretation should be viewed as tentative. Our assumption of one-dimensionality overestimates the motion at distant points, although this effect is small for points close to the dyke, which dominate our values of U_0 and κ . Ignoring the effect of older transients underestimates the motion at distant points. The radial component of the deformation field apparent in Fig. 1b results from the real two-dimensionality of the dyke. We assume thinness and perfect elasticity for the upper layer, newtonian behaviour for the lower layer and lateral homogeneity. These approximations probably have a significant effect on the value that we calculate for the diffusivity, κ , and thus viscosity, η . A model incorporating viscoelasticity and finite layer thicknesses would result in modified values.

Of the three stages of the 'seismic cycle' (strain accumulation between events, co-seismic elastic step and post-seismic transient), the strain transient is the least well observed. But transient behaviour contains unique information about the structure and rheology of the Earth and its response to stress changes. In addition, it influences the temporal interactions of earthquakes. Transient stress diffusion is a physical mechanism by which the stress changes accompanying an earthquake or rifting episode are redistributed to adjacent regions and contribute eventually to triggering other earthquakes. Statistical analyses of the spatial and temporal correlations between earthquakes have had little success in predicting their behaviour. A physical model that incorporates both co-seismic elastic and delayed transient stress redistribution may be a better prediction tool.

Observations of transients from spreading plate boundaries are particularly difficult to acquire because most of the world's rift system is on the ocean floor. A similar but smaller dyke intrusion event in 1978 in the rift system of Afar, East Africa, was studied geodetically^{24–26}. The deformation following the intrusion is compatible with that from Northeast Iceland. The network in Afar, extending to 37 km from the rift, was however too narrow to detect any but the quasi-linear central part of the

stress diffusion curve, and the observations were interpreted as a steady state process controlled by the plate motion. Reinterpretation of the Afar observations in terms of transient stress diffusion would be expected to yield insights into the structure and rheology of this area. □

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Ridge segmentation, faulting and crustal thickness in the Atlantic Ocean

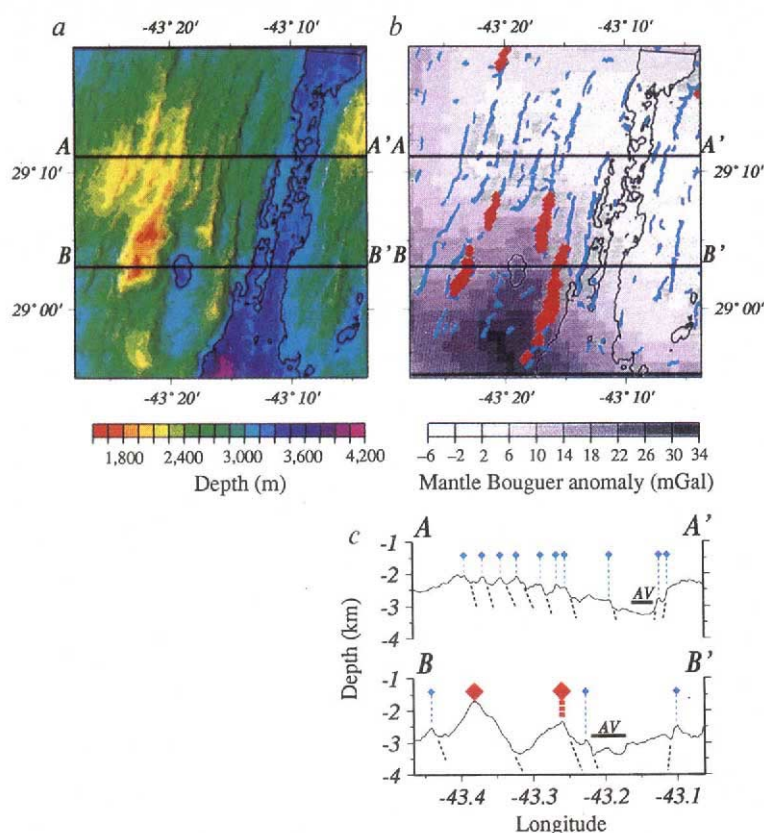
Peter R. Shaw

Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543, USA

THE Mid-Atlantic Ridge (MAR) between the Kane and Atlantis fracture zones consists of segments 20–85 km in length¹; bull's-eye patterns in the mantle Bouguer gravity anomaly field² centred on several segments associated with narrow rift valleys³ have been interpreted as centres of strong mantle upwelling and thick crust. Here I present a map of normal faults inferred from $\sim 5 \times 10^4 \text{ km}^2$ of Sea Beam bathymetry⁴ along the MAR between 28° N and the Atlantis fracture zone. Faults are mapped both on- and off-ridge using a criterion that distinguishes them from volcanic topography. Abyssal hill lineations seem to form at the rift valley walls through the growth of the normal faults; towards ridge segment ends these faults are more widely spaced, with larger throws than those at segment centres, and often define the boundaries of the gravity bull's-eyes. These two different faulting styles, which probably reflect changes in lithospheric strength, are preserved into the rift mountains. Large-throw faults are highly correlated with the mantle Bouguer highs, suggesting that amagmatic extension on the large faults contributes to the crustal thinning inferred towards the segment ends.

To distinguish faults from constructional volcanism, both of which can create steep slopes, I measure topographic curvature

FIG. 1 Mapping of faults near the Mid-Atlantic Ridge. *a*, Shaded relief map (Sun from northwest) of topographic sample that includes the median valley, roughly defined by the 3,200 m contour (black contour) and a portion of the rift mountains. Profile A–A' samples the centre of this ridge segment; B–B' samples near the end of the segment. *b*, Relation between faults and residual mantle Bouguer anomaly². Blue symbols: faults mapped using a window radius of 0.45 km, chosen to highlight the smallest faults seen in the data. Red symbols: 2.2-km-radius window highlights exclusively the largest faults and similar large features. Grey shades: residual mantle Bouguer anomaly; 3,200-m contour is repeated. *c*, Cross-section and interpretation of the two profiles A–A' and B–B'; locations of small- and large-scale fault corners also indicated. The vertical scale is exaggerated ($\times 4$). Nearly all the rugged topographic ridges are associated with normal faults, as identified by their sharp edges: these only form starting at the edges of the rift walls. Neovolcanic ridge within median valley^{1,7} does not create any highlighted edges.



which detects the sharp linear edges unique to the upthrust footwall of normal faults. The analysis fits a quadratic surface to topography within a small circular window which is scanned across the study region. Sharp ridges and peaks are flagged by marking the locations where the most negative principal curvatures fall below a threshold, which is readily adjusted to highlight fault edges while excluding constructional volcanism such as within the median valley. I investigate the scale dependence of fault distributions by selecting two representative radii of the analysis aperture, 0.45 km and 2.2 km, corresponding to the span of fault scales observed in the region. Each window detects only those faults whose spatial scale roughly matches the aperture, thus providing a means of classifying the faults by their size.

A plot of fault locations reveals clusters of subparallel linear forms, interpreted as linear fault edges from their cross-sectional topographic profile (Fig. 1); smaller, unresolved normal faults

are almost certainly present^{5,6}. Most of the abyssal hill lineations apparent in the shaded relief map are identified as faults when analysed at the 0.45-km scale. These sharp linear edges are generally not associated with the neovolcanic ridge or other features within the median valley^{1,7} but are initiated at the axial valley walls. This finding clarifies the role of faulting in the formation of abyssal hills at slow-spreading ridges^{5,8–12} by linking lineations observed in the rift mountains with their formation location and mode. Most of the rugged off-axis lineated topographic relief characteristic of the Atlantic seems to be produced primarily by the growth of large normal faults beginning at the rift walls, rather than by constructional volcanism^{11,13} or by the tectonic splitting of the axial volcanic ridge in the centre of the median valley¹⁰. Observed volcanic constructs at the edges of hanging walls^{5,12} would indicate that the constructs provide preferred sites for fault formation, but only at the median valley wall¹².

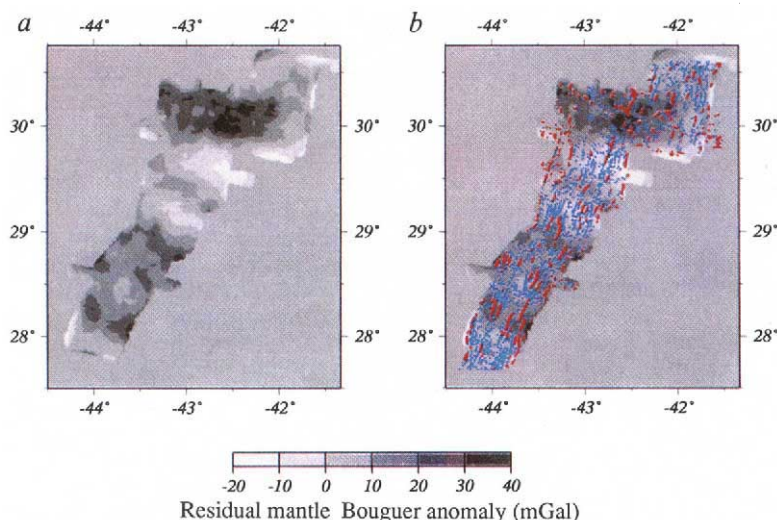


FIG. 2 Relation of faulting to ridge segmentation and mantle upwelling. *a*, Residual mantle Bouguer anomaly (RMBA) from ref. 2; light shades indicate RMBA lows, inferred centres of mantle upwelling and thick crust; dark grey indicates thin crust. *b*, Locations of fault corners found using the 0.45-km-radius analysis window (blue) and 2.2-km window (red). Large faults predominantly occur in clusters over RMBA highs; continuation of large faults off-axis implies that tectonic differences between segment centres and ends is persistent; ribbons of similarly tectonized crust associated with segment ends are carried off into rift mountains.

Sempéré *et al.*³ found variations in linearity and throw in the active rift-bounding faults, correlating with fault location within the segments. In this study I map fault locations at two spatial scales, revealing a marked difference in faulting style that extends off-axis into the rift mountains: the centre (for example, profile A-A') of the segment in Fig. 1 is dominated by closely spaced, small-throw faults (blue) whereas the end (B-B') is characterized by more widely spaced, larger-throw faults (red symbols). This difference in fault spacing implies that faults occur more readily at segment centres than towards segment ends, possibly reflecting a weaker lithosphere at segment centres. Although abundant magma near the segment centre may periodically partially cover the faults and reduce their apparent throw, the difference in the fault spacing indicates structural differences that go beyond volcanic covering.

Associated with these large-throw faults in profile B-B' is a large topographic rise and valley near 29° 04' N, 43° 20' W (paired orange and blue regions); at the same location is a significant local maximum (dark grey shades) in the residual mantle Bouguer anomaly² (RMBA), a probable indicator of thin crust in this area. The close coincidence of these features suggests that such faults, with vertical offsets exceeding 1 km, can produce considerable local crustal thinning and directly contribute to the RMBA high as well as a surface topographic expression. Penetration of the crust by large near-ridge faults has been suggested from analysis of earthquakes associated with slow-spreading ridges¹⁴⁻¹⁸.

Figure 2 pursues this association between large faults and RMBA where the RMBA field is available², between latitudes 28° N and 30° 30' N. A correlation is apparent between the RMBA and large faults throughout this area: large faults cluster near the ends of segments, closely matching the RMBA highs and defining the edges of the RMBA bull's-eyes. These large faults (spacing ~4–8 km, 0.8–1.2 km vertical displacement) are curved and continuous for only ~5–10 km; the smaller-scale faults (spacing 0.8–2 km, vertical displacement 0.2–0.6 km) are more common near centres of ridge segments, and are longer (up to 40 km) and more linear than the large faults.

The correlation between fault locations and RMBA gravity highs is quantified by analysing the RMBA values for the region south of 29° 50' (excluding the Atlantis fracture zone). To test whether the distribution of RMBA values at the locations of large faults is different from the RMBA values over the whole region, I have calculated the two cumulative probability distributions (Fig. 3). The difference of 0.2 between these distributions is statistically significant using the Kolmogorov-Smirnov test¹⁹, implying a genetic relation between the large faults and RMBA gravity highs.

Large-throw faults provide a mechanism for contributing to

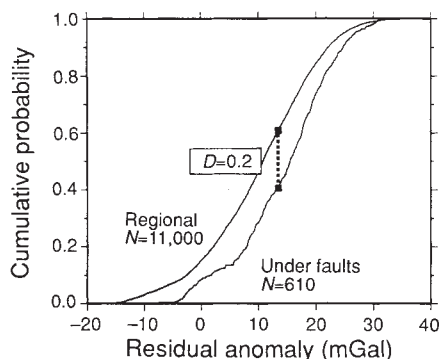


FIG. 3 Cumulative histograms (scaled between 0 and 1 so that they approximate cumulative probability distributions) of RMBA field for all the mapped RMBA south of 29° 50' and the RMBA values directly over the large faults. These distributions differ by up to $D=0.2$, which is significant at the 99% confidence level using the Kolmogorov-Smirnov test¹⁹. This result quantifies the strong association of large faults with RMBA highs.

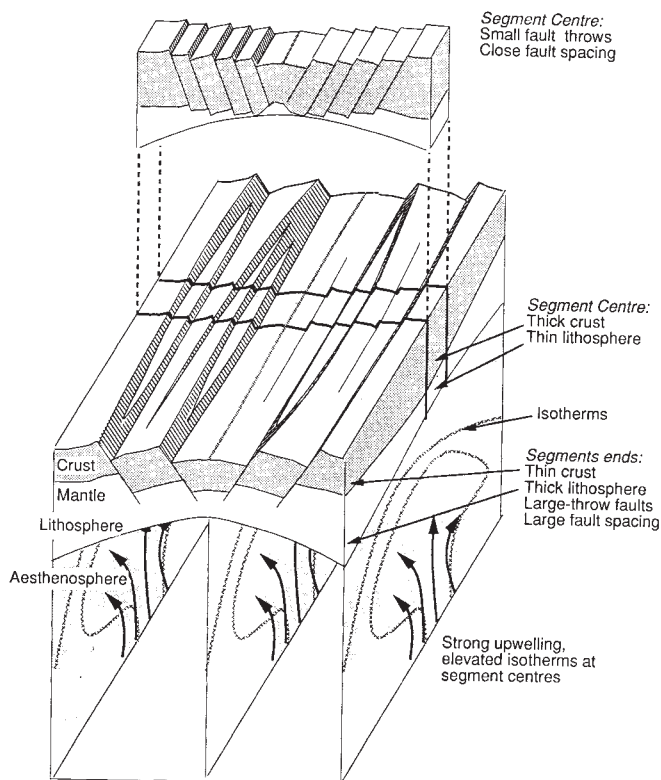


FIG. 4 Geological interpretation. Conceptual view with cross-sections through the centre and end of a ridge segment associated with a RMBA bull's-eye low. Cross-section through segment centre (cutaway at top) portrays numerous closely-spaced, small-throw faults above strong mantle upwelling. Section through end of segment (at bottom) shows large-throw faults resulting from amagmatic extension, and smaller magma supply. These large faults seem to contribute to crustal thinning at ridge segment ends. Isotherms are inferred to be shallower toward ridge segment centres, so the lithosphere is thinner here than at segment ends. Observed variations in fault spacing and throw reflect this difference in lithospheric strength.

variations in crustal thickness toward the ends of non-transform ridge segments^{2,20,21} (Fig. 4). If the melt supply within a segment is greater at the centre but the rate of extension spatially uniform, the segment ends will be thinned more by amagmatic extension than will centres.

Additionally, more available melt and shallower isotherms toward segment centres would reduce the strength of the lithosphere, inhibiting the sustained growth of faults by allowing new faults to form easily. This result is consistent with conclusions of studies of the MAR rift valley in this region from teleseismic earthquakes²² and rift valley morphology^{1,3}. Amagmatic crustal stretching within the rift valley has been inferred toward segment ends in this region^{1,3} and from submersible studies just south of the Kane fracture zone^{23,13}. Asymmetries of the rift valley³, notably high inside-corner topography²⁴⁻²⁶, will be superimposed on these within-segment differences.

The continuous trail of large faults from the active zone at the ridge axis into the ridge mountains (Fig. 2) indicates that the tectonic differences between the centre and ends of a segment are persistent in time, rather than representing different stages of a spatially uniform cycle. Such spatial variations in tectonic styles would create ribbons of similar crust off-axis. □

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Importance of habitat saturation and territory quality for evolution of cooperative breeding in the Seychelles warbler

Jan Komdeur*

Department of Zoology, University of Cambridge, Cambridge CB2 3EJ, UK

COOPERATIVE breeding, which often involves young remaining on their natal territory and helping their parents to raise subsequent broods^{1–3}, is mostly explained by habitat saturation: young are constrained from becoming independent breeders by a shortage of breeding territories^{2,4}. Here I present two lines of evidence against this hypothesis for the cooperatively breeding Seychelles warbler *Acrocephalus sechellensis*. First, territory quality has a significant effect on dispersal: vacancies arising on territories are mostly filled by prebreeding birds from territories of the same or lower quality. Second, individuals that delay reproduction in high-quality territories, but which eventually breed there, have greater lifetime fitness than those that disperse at one year of age and breed immediately in lower-quality territories. These results support the ‘benefits of philopatry’^{5,6} hypothesis, which emphasizes the lifetime inclusive fitness benefits from staying at home. The transfers of warblers to unoccupied islands was the strictest experimental test of this hypothesis. At first there was no cooperative breeding, but as all high-quality areas became occupied, young birds born on high-quality territories began to stay as helpers, rather than occupying breeding vacancies on low-quality territories. Therefore habitat saturation and territory quality are both involved in the evolution of cooperative breeding.

At one time the total world population of the endemic Seychelles warbler was reduced to 26 individuals, entirely confined to Cousin Island (29 Ha)⁷. Following long-term management of Cousin by the International Council for Bird Preservation since 1968^{7,8}, the warbler made a spectacular recovery (Fig. 1a). Since 1982, the population has fluctuated around 300 birds, suggesting that this is the maximum carrying capacity of the island. Helping was first observed in 1973 in a few rich territories⁹ (at about the time the number of territories

reached saturation level), but since 1982 it has been widely observed all over the island, suggesting that habitat saturation was the main cause of cooperative breeding (Fig. 1b).

On Cousin, all 115–123 groups (310–400 birds) were checked fortnightly for breeding activity from January 1986 to September 1990. Data were based on individually colour-ringed birds. Very few warblers bred at age one. Virtually all offspring that failed to become breeders remained on their natal territory, from which they forayed into the neighbourhood searching for openings in the breeding population. Helpers aided in territory defence, predator mobbing, nest-building, incubation (only females) and feeding young.

As nest-predators were evenly distributed over the island¹⁰, and nest sites were plentiful¹¹, territory quality was measured in terms of insect prey available¹¹ (Fig. 1b).

Foraging efficiency was higher in high-quality territories¹¹, leading to increased reproductive success and survival (Table 1). As a consequence, fewer vacancies arose on high-quality territories (of 96 vacancies, 59.4% arose on low-quality territory, 30.2% on medium-quality territory and only 10.4% on high-quality territory). The options for young warblers were either to fill a vacancy in a low-quality territory, where the probability of immediate breeding was higher, or to remain as a non-breeder in a territory of higher quality. Because territory quality affected reproductive success as well as survival rates, lifetime pay-offs from either dispersing or staying had to be considered for each territory category.

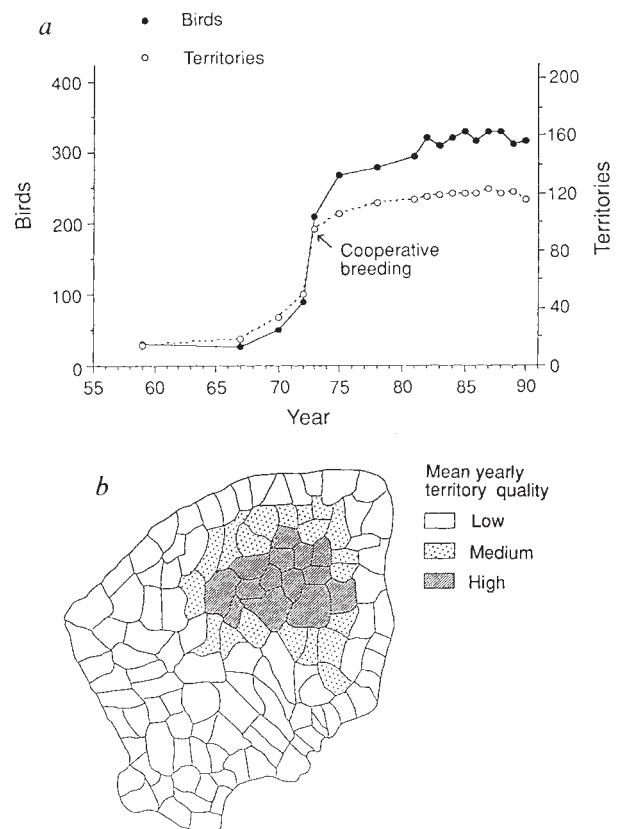


FIG. 1 Increase in Seychelles warbler numbers and territories since 1959¹¹ (a), and mean yearly quality of Seychelles warbler territories on Cousin Island (1986–1990; b). Territory quality was calculated from three measures: mean yearly territory size, mean foliage cover for each plant species and mean insect totals for each plant species per unit leaf area (Table 1)¹¹, and divided into three categories: low-, medium- and high-quality territory. The island was completely covered by territorial groups with no empty spaces. The central area consisted of high-quality territories; high vegetation cover, little wind and therefore many insects. In contrast, the coastal area consisted of many low-quality territories, as wind-driven saltspray caused defoliation, which resulted in low insect numbers.

* Present address: National Environmental Research Institute, Department of Wildlife Ecology, Kalø, Grenåvej 12, DK-8410 Rønde, Denmark.

TABLE 1 Effects of territory quality on the Seychelles warbler on Cousin Island (1986–1990)

	Territory quality†			Statistic‡
	Low	Medium	High	
Yearly reproductive success per unaided pair§	0.19 ± 0.12 (286)	0.51 ± 0.19 (38)	0.85 ± 0.21 (28)	$H=101.04^*$
Yearly reproductive success per group (V)§ (pair plus helpers)	0.22 ± 0.13 (365)	0.85 ± 0.21 (60)	1.27 ± 0.36 (55)	$H=161.35^*$
Mean group size	2.4 ± 0.21 (365)	2.9 ± 0.91 (60)	3.7 ± 0.93 (55)	$H=110.12^*$
First-year survival (s)	0.30 (103)	0.67 (23)	0.86 (22)	$\chi^2=28.85^*$
Annual adult survival (s)	0.76 (156)	0.88 (64)	0.91 (48)	$\chi^2=7.82^{**}$
Mean adult life expectancy (z)	2.7	5.4	7.4	

The table shows the effect of territory quality on breeding success and mean group size ($\pm$ standard deviation; numbers in parentheses are territory years), first-year survival (the probability of surviving to age of one year) and annual adult survival (probability of surviving to the next year, starting at age of one year) (numbers in parentheses are bird years based on colour-ringed individuals), and mean adult life expectancy (the length of time after which 50% of the population alive at one year will have died). A pair consists of one male and one female. There was no emigration from the island, so birds that disappeared could be reliably scored as dead. Seychelles warbler pairs occupying high-quality territories fledged significantly more young per year than pairs on lower-quality territories. First year survival and annual adult survival increased significantly with territory quality. Mean adult life expectancy was therefore also significantly correlated with territory quality.

† Territory quality = $(a \sum_{i=1}^{12} (c_i i_x)) / 100$, where a is mean yearly territory size (hectares), c_i is mean yearly foliage cover for plant species x , and i_x is mean monthly insect totals for plant species x per unit leaf area (one square decimetre) per year.

‡ H , Kruskal-Wallis test; χ^2 , test for comparing more than two proportions; * $P < 0.001$, ** $P < 0.025$.

§ Yearly number of fledglings reaching one year of age.

|| Survival $s = (b/a)^{12/n}$, where a = number of ringed birds present in year y , b = number of ringed birds still present in year $y+1$ and n = number of months between counts in year y and year $y+1$.

Option 1: dispersal and breeding. The lifetime fitness (LF_b) realized by dispersing and breeding independently in year a is given by:

$$LF_b = \sum_{x=a+1}^z (p V_x r_{hy}) \quad (1)$$

where z is mean adult life expectancy, p is the probability of successful dispersal and establishment as a breeder, V_x is the number of yearlings produced at age x , and r_{hy} is the coefficient of relatedness between helper 'h' (now a breeder) and its own young 'y'. Breeders dispersed only once (out of 137 ringed adults, only one (0.7%) changed breeding territory) and when they filled a vacancy, immediate breeding status was always acquired ($n=96$).

Option 2: staying and helping. Removal experiments showed that helpers could raise the reproductive success of their parents¹¹. The production of additional, related individuals beyond what the parents might have produced, added an indirect component to lifetime reproductive success^{2,5,12} estimated as

$$LF_h = \sum_{x=a+1}^z ((V_n - V_{n-1})_x r_{hby}) \quad (2)$$

where $(V_n - V_{n-1})$ is the mean increase in yearlings produced per year for each change in group size, weighed by the number of groups of that size observed during the study period, and r_{hby} is the coefficient of relatedness between the helper 'h' and the young of the breeder 'by' it helped to rear. Of all helpers ($n=121$), 94.2% were grown offspring of both breeders ($r_{hby} \approx 0.50$).

Direct fitness benefits (1) together with indirect fitness benefits (2) yield the overall expected lifetime reproductive success. Immediate breeding benefits on a given quality territory were always greater than helping benefits (Fig. 2). A one-year-old

warbler, however, could produce more offspring over its lifetime by remaining in a high-quality territory for several years as a helper and then breeding, than it could by dispersing and breeding immediately in a lower-quality territory (Fig. 2).

These lifetime reproductive success payoffs predicted the observed dispersal behaviour. Yearlings born on high-quality territories were more likely to remain at home as helpers (92.7%; $n=41$) than those born on medium-quality territory (69.2%; $n=26$) or low-quality territory (28.6%; $n=56$). The effect of territory quality on dispersal decisions was also revealed by comparing the origins of individuals that became breeders in each territory type: 89.5% of breeders in high-quality territory were born in high-quality territory ($n=38$); for medium-quality territory and low-quality territory this figure was 61.5% ($n=26$) and 92.7% ($n=41$), respectively. These analyses indicate that birds acting as helpers in high-quality territory were more likely to wait for a vacancy in a high-quality territory, rather than dispersing to lower-quality territories as breeders, than were individuals from medium- and low-quality territories.

The transfers of 29 individuals to the unoccupied Aride Island (68 Ha) in September 1988^{11,13} and of 29 birds to the unoccupied Cousin Island (26 Ha) in June 1990¹¹, provided an experimental test of the 'habitat saturation' hypothesis. All 38 vacancies on Cousin Island created by removals were filled immediately, some within hours, by previous helping and/or one-year-old birds. Most vacancies in high-quality territories were filled by birds born in high-quality territories (92.9%; $n=14$), suggesting that young from high-quality territories were better competitors for prime breeding territories. Medium-quality vacancies were only filled by birds from medium- and low-quality territories ($n=11$), and low-quality vacancies only by birds born in low-quality territories ($n=13$). These results were not biased by a skew in the distribution of surrounding alloparents on different quality territories¹¹.

At first, the mean age at which juveniles left their natal territories was 4.0 months on Aride ($n=85$) and 4.2 months on

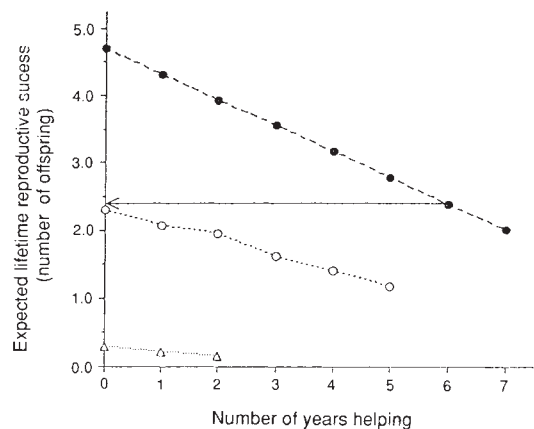


FIG. 2 Expected lifetime reproductive success, in different quality territories, of Seychelles warbler yearlings that helped for a different number of years before breeding¹¹ (equations (1) and (2); Table 1). The following assumptions were made: (1) All individuals survived to one year old, the point at which Seychelles warblers decided for the first time whether to disperse or remain in a group. (2) Group size during breeding was the average for territories of that quality. (3) A bird leaving its natal group was always able to breed ($P=1$). (4) No breeding status was achieved by the helper within the bird's original group ($r_{hby}=0.5$). A bird that helped for seven years on a high-quality territory (●) could still expect to produce 6.7 times more offspring equivalents³ than a bird breeding immediately in a low-quality (△) territory (2.01 versus 0.30), and a bird could help for six years before breeding in a high-quality territory and still expect to produce on average the same number of offspring equivalents as a bird that dispersed and bred at one year in a medium-quality (○) territory (2.39 versus 2.30).

Cousin ($n=14$), eight weeks after reaching independence; compared with 23.3 months on Cousin ($n=93$, $P<0.001$). The mean age at which young bred was 8.1 months on Aride ($n=61$) and 8.3 months on Cousin ($n=4$), again much sooner than on Cousin (47.3 months, $n=44$, $P<0.001$). None of the 105 young, old enough to be a helper (91 and 14, respectively) acted as a helper on their natal territories. It is very clear then that habitat saturation is the pre-eminent cause of cooperative breeding on Cousin.

Two years after the transfer to Aride, and a little more than one year after the transfer to Cousin, all high-quality areas on both islands have become occupied and young birds born on high-quality territories have begun to stay as helpers, even though there is still abundant space for them in lower-quality areas to establish territories (refs 11 and 14, respectively). It can be concluded that habitat saturation and territory quality are both involved in the evolution of cooperative breeding. That territory quality should be important to dispersal options by offspring was also emphasized by the 'marginal habitat' model¹⁵, a refined version of the 'habitat saturation' hypothesis. □

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Human infection by genetically diverse SIV_{SM}-related HIV-2 in West Africa

Feng Gao*, Ling Yue*, Albert T. White†, Peter G. Pappas‡, Joseph Barchue§, Aloysius P. Hanson§, Bruce M. Greene†, Paul M. Sharp||, George M. Shaw* & Beatrice H. Hahn*

Department of Medicine, * Divisions of Hematology/Oncology, † Geographic Medicine, and ‡ Infectious Diseases, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA
§ Liberian Institute for Biomedical Research, Robertsfield, Liberia
|| Department of Genetics, Trinity College, Dublin 2, Ireland

OUR understanding of the biology and origins of human immunodeficiency virus type 2 (HIV-2) derives from studies of cultured isolates from urban populations experiencing epidemic infection and disease^{1–8}. To test the hypothesis that such isolates might represent only a subset of a larger, genetically more diverse group of viruses, we used nested polymerase chain reactions to characterize HIV-2 sequences in uncultured mononuclear blood cells of two healthy Liberian agricultural workers, from whom virus isolation was repeatedly unsuccessful, and from a culture-positive symptomatic urban dweller. Analysis of *pol*, *env* and long terminal repeat regions revealed the presence of three highly divergent HIV-2 strains, one of which (from one of the healthy subjects) was significantly more closely related to simian immunodeficiency viruses infecting sooty mangabeys and rhesus macaques (SIV_{SM}/SIV_{MAC}) than to any virus of human derivation. This subject also harboured multiply defective viral genotypes that resulted from hypermutation of G to A bases. Our results indicate that HIV-2, SIV_{SM} and SIV_{MAC} comprise a single, highly diverse group of lentiviruses which cannot be separated into distinct phylogenetic lineages according to species of origin.

In 1989 we conducted a limited seroepidemiological survey in Liberia, West Africa, to estimate the prevalence of HIV-1 and HIV-2. Serum samples were collected from 372 healthy adults living in remote villages of northern Liberia, 944 rubber plantation workers attending outpatient clinics in rural areas of central Liberia, and 366 adults from the capital city of Monrovia and surrounding urban areas. Specimens were first screened by a recombinant HIV-1/-2 combination enzyme-linked immunosorbent assay (ELISA) and subsequently confirmed by western immunoblots specific for HIV-1 and HIV-2. Three individuals,

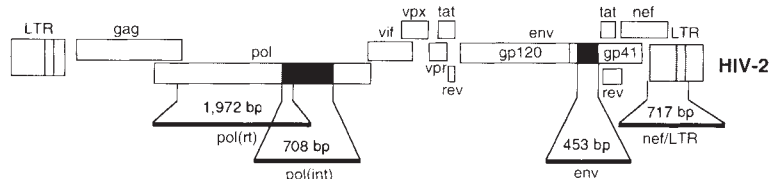
one from Monrovia with frank AIDS and two from surrounding areas with unknown clinical status, were seropositive for HIV-1. Five other individuals, all healthy inhabitants of rural or remote villages, were seropositive for HIV-2. Two of these individuals (F0784 and 2238) could be relocated for further serologic testing, virus culture and analysis by polymerase chain reaction (PCR). Both were male workers from rubber plantations, ages 46 and 47. Their physical examinations were normal, and they had no history of sexually transmitted disease, chronic illness, blood transfusion or homosexuality. For comparison, a third HIV-2-seropositive but symptomatic man from Abidjan, Côte d'Ivoire was also studied (7312A). He was 32 years old, had lymphadenopathy, cutaneous anergy and recurrent skin abscesses, and reported frequent sexual encounters with urban prostitutes. Blood specimens from all three subjects were obtained on two separate occasions and processed for virus isolation and PCR analysis^{9,10}. Mononuclear cells (PBMCs) were cultured alone, in combination with normal donor lymphocytes and macrophages, and with immortalized T-cell lines (Molt4 clone8, CEM × 174, H9, SupT1). HIV-2 was successfully isolated from subject 7312A on each of two occasions but not from subjects F0784 and 2238, despite optimal cell growth and viability.

To determine the genetic identity of viral sequences in subjects F0784, 2238 and 7312A, we used a highly sensitive nested PCR technique to amplify viral sequences directly from uncultured PBMC DNA^{10,11}. Using primer pairs designed according to HIV-2/SIV_{MAC}/SIV_{SM} consensus sequences¹², 708-base-pair (bp) *pol* and 453-bp *env* fragments were amplified (Fig. 1) and sequences corresponding to a total 34,770 nucleotides were determined. This analysis revealed viral mixtures, or quasi-species¹³, of varying complexity for each subject (Fig. 2). F0784 harboured the largest number of variants, most of which represented defective (prematurely terminated) viral genomes that resulted from G-to-A hypermutation¹⁴. Such G to A changes, which were found in two different genes (*pol* and *env*) and in blood samples obtained four months apart, accounted for 66% to 87% of all nucleotide substitutions in F0784 and resulted in sequence differences among individual *env* clones that were as high as 11.5%. *Env* and *pol* regions from 2238 and 7312A exhibited much less intrastrain variability (<0.3%) and no G-to-A hypermutation, which together with our previous studies on SIV_{AGM}¹⁰, indicated that *Taq* polymerase or sequencing errors did not contribute significantly to the G-to-A changes observed in F0784.

To determine the phylogenetic relationships among HIV-2_{F0784}, HIV-2₂₂₃₈, HIV-2_{7312A}, and other HIV-2/SIV_{SM}/SIV_{MAC} strains, we constructed evolutionary trees for their *pol* and *env*

FIG. 1 Location of HIV-2 sequences amplified from uncultured PBMC DNA. Dark shaded areas highlight *pol* (integrase, int; 708 bp) and *env* (453 bp) fragments amplified from all three study subjects (F0784, 2238, 7312A). Lighter shaded areas indicate *pol* (reverse transcriptase, rt; 1,972 bp) and *nef*/LTR (717 bp) fragments amplified only from subject F0784.

METHODS. Nested PCR amplification, cloning and sequencing have been described¹⁰. Primer pairs were designed according to HIV-2/SIV_{MAC}/SIV_{SM} consensus sequences¹² and are numbered according to HIV-2_{ROD} (ref. 1): *env* A 5'-GCTAGGGTCTTGGGTTTC-TCGCAGCAGCAGG-3' (nucleotides (nt) 7,691-7,723); *env* B 5'-CAAGAGCGT-ATCAGCTGGCGGATCAGGAA-3' (nt 8,415-8,444); *env* C 5'-GGGATAGTGC-AGCAACAGCAACAGCTGTTG-3' (nt 7,782-7,811); *env* D 5'-GGGAGGGGAA-GAGAACACTGGCCTATA-3' (nt 8,265-8,291); *pol* A (same as *pol* C) 5'-AATATACTAGTAGATTCACAATATGT-3' (nt 3,857-3,882); *pol* B 5'-CTGCC-TTCTCTGAAATAGACCCGAAA-3' (nt 4,723-4,749); *pol* D 5'-CTTCTTTTAA-AATTCATGCAATGAAGTCC-3' (nt 4,591-4,620); reverse transcriptase (RT) A 5'-ATAGTAGGTGGAATAGGGGTTTCATAAATA-3' (nt 2,219-2,249); RT B 5'-TGAAGTGAAGTTGGCACCCTGTCTGTGTG-3' (nt 4,397-4,426); RT C 5'-ATGACAGGGGATCCCCAATCAATATTTTG-3' (nt 2,309-2,339); RT D 5'-CTTCTGTTTCTGTGGAATGACTTCTGCT-3' (nt 4,312-4,341); *nef* A 5'-AATATGGATGTGATGATTCAGATGATGATGAC-3' (nt 8,812-8,844); LTR B 5'-AAGCAGAAAGGGTCTAACAGACCAGGT-3' (nt 9,739-9,767); *nef* C 5'-TGGCAATAGATATGTACATTTTATAAA-3' (nt 8,900-8,927); LTR D 5'-CCAGG-



CGGCGACTAGGAGAGATGGGAGCAC-3' (nt 9,682-9,711). Amplification conditions were 94 °C, 1.5 min; 45 °C, 1.5 min; 55 °C, 1.5 min; 35 cycles for *env* primer pairs; 94 °C, 1 min; 40 °C, 1.5 min; 70 °C, 2 min; 30 cycles for *pol* (integrase) primer pairs; 94 °C, 1.5 min; 40 °C, 1 min; 72 °C, 4 min; 30 cycles for *pol* (reverse transcriptase) primer pairs, and 94 °C, 1.5 min; 40 °C, 1.5 min; 72 °C, 2 min, 30 cycles for *nef*/LTR primer pairs. The number of individual recombinant clones sequenced were as follows: F0784A *env* (11/89 PBMC), 21 clones; F0784C *env* (3/90 PBMC), 16 clones; F0784C *pol* integrase (3/90 PBMC), 5 clones; F0784C *pol* RT (3/90 PBMC), 4 clones; F0784C *nef*/LTR (3/90 PBMC), 3 clones; 2238 *env* (5/89 PBMC), 17 clones; 2238 *pol* (5/89 PBMC), 5 clones; 7312A *env* (9/90 PBMC), 3 clones; 7312A *env* (9/90 cultured), 1 clone. 7312A *pol* was sequenced directly from amplified products. A complete compilation of primary sequence data is available from GenBank/EMBL (accession numbers M87069-M87145) and the HIV database (Los Alamos National Laboratories).

sequences using both maximum parsimony¹⁵ and neighbour-joining¹⁶ methods (Fig. 3). In both *pol* (Fig. 3a) and *env* (Fig. 3b) regions, seven previously reported ('prototypic') isolates of HIV-2 clustered in a closely related group. In contrast, the three strains reported here branched quite differently. HIV-2₂₂₃₈ was most closely related to a single divergent HIV-2 isolate, HIV-2_{D205} (ref. 7), although these two viruses differed from each other to a greater extent than was typical for prototypic HIV-2 strains. HIV-2_{7312A} appeared to have a mosaic genome: its *pol* sequence was most closely related to HIV-2_{D205} (Fig. 3a), yet its *env* sequence clustered with the prototypic HIV-2 isolates (Fig. 3b). These discordant relationships between the HIV-2_{7312A} *pol* and *env* regions were strongly supported by bootstrap analyses¹⁷ and probably reflect recombination between phylogenetically divergent strains.

The most striking finding from the phylogenetic analyses was that HIV-2_{F0784} *pol* and *env* sequences clustered with the simian viruses, SIV_{SM} and SIV_{MAC}, rather than with other HIV-2 strains. This relationship was found in the majority, although less than 95%, of bootstrap samples, and so to substantiate the phylogenetic position of HIV-2_{F0784} we amplified and analysed two additional regions of its genome: a 1,972-bp *pol* fragment encoding reverse transcriptase and a 717-bp *nef*/long terminal repeat (LTR) fragment (Fig. 1). Phylogenetic analyses of the reverse transcriptase fragment using both maximum parsimony and neighbour-joining algorithms (Fig. 3c) showed clustering of HIV-2_{F0784} with the SIV lineage in more than 99% of bootstrap samples. The *nef*/LTR region was chosen for analysis because it encompasses a 40-44-bp 'signature' sequence that is present in all published clones of HIV-2 (including HIV-2_{D205}) but is absent from all known SIV_{SM}/SIV_{MAC} sequences¹². Like the SIV genomes, HIV-2_{F0784} lacks this signature sequence (Fig. 4), which therefore can no longer distinguish between viruses that infect man and those that infect monkeys.

The results of our phylogenetic analyses, together with structural features unique to HIV-2/SIV_{SM}/SIV_{MAC} genomes^{1,12,18} (that is, the presence of both *vpr* and *vpx* genes, and duplication of the LTR *trans* activation response (TAR) element), indicate that HIV-2 in man and SIV in mangabeys and captive macaques represent members of a single, albeit genetically diverse, group of viruses. Although the evolutionary origins and transmission patterns of this virus group remain to be defined, there is mounting evidence that the sooty mangabey is a natural reservoir and that human infection probably represents a zoonosis (a disease communicable from animals to man under natural con-

ditions¹⁹). First, roughly 10% of wild-caught sooty mangabeys in Côte d'Ivoire and Liberia are infected with SIV_{SM} (ref. 20; and P. Fultz, personal communication). Second, the natural habitat of mangabeys coincides with the geographic pattern of HIV-2 endemicity and close contact between mangabeys and man (and between mangabeys and captive macaques) is well documented^{18,20-22}. Third, SIV_{SM} generally fails to cause disease in mangabeys^{20,23} but is highly pathogenic in macaques^{21,22,24}, as is the case for many zoonotic infections that cause less severe or even no disease in their natural hosts¹⁹. Definitive proof of zoonotic infection will require direct epidemiological and genetic evidence of SIV_{SM} transmission from an infected mangabey to man. Short of this, the identification of an HIV-2 strain (F0784) that is phylogenetically more closely related to simian than to human immunodeficiency viruses (Fig. 3) provides the strongest evidence yet for simian/human cross-species transmission.

It is notable that most previously studied isolates of HIV-2 were obtained by virus culture from individuals who were generally symptomatic and resided in areas where HIV-2 was spreading epidemically¹⁻⁸. In contrast, HIV-2_{F0784} and HIV-2₂₂₃₈ could not be cultured (despite repeated attempts) from healthy inhabitants of rural areas where HIV seroprevalence was low and AIDS had not been recognized clinically. This raises the possibility that certain naturally occurring strains of HIV-2 may replicate less efficiently and cause less virulent infection. It is of interest that the majority of HIV-2 sequences from subject

FIG. 2 Intrastrain variability and G-to-A hypermutation in HIV-2. An alignment of 453-bp *env* sequences is shown. Individual clones are compared to a predominant, non-truncated clone with dashes indicating sequence identity and a dot indicating a single nucleotide deletion. Individual clone designations, number of clones with identical sequence versus total number of clones analysed, and the number of defective clones (def) with premature truncations are indicated on the right. Asterisks indicate premature stop codons resulting from G-to-A hypermutation.

METHODS. HIV-2 *env* fragments were amplified, cloned, and sequenced as described in Fig. 1. F0784 *env* sequences were amplified from two sequential blood specimens (11/89 and 3/90) on three different occasions resulting in 21, 10 and 6 recombinant M13 clones, as shown. Short-term PBMC cultures of subject 7312A were also analysed as indicated (cultured). Individual sequences were aligned using the program EUGENE (Baylor College of Medicine).

F0784 env

[illegible][illegible][illegible]

2238 env

	Accession	Gene	Protein	Species	Strain	Position	Length	Score	E-value
5/89	GACCTGGTCAAAAGACAACAGGAAATGTTGGCGCTGACCGCTGGGGAACATAAAAGCTCCACAGCTACAGTCTACGCCACGAGAAGTACCTAAAGACCAAGCAAAATAAATTCAGGGGATGGCCTTAGACAGGTTGCCACACATAAspValValLysArgG.nGlnGluMetLeuArgLeuThrValTrpGlyThrLysAsnLeuGlnThrArgValThrAlaIleGluLysTrpLeuLysAspGlnAlaLysLeuAsnSerTrpGlyCysAlaPheArgGlnValCysHisThrT					B10	12/17		
	-----G-----					B3	3/17		
	-----G-----					B1	1/17		
	-----G-----					B2	1/17		
5/89	CTGTCCCA1GGGTGAACGACAGATGACGCCAGAGTGCCAAAACATGACTGGCAACAATGGGAAAAACAGATTGCTCTTTCGACGGATAATATCACACAGCTCTTAGAGCAAOCTCAAATACAGCAGCAGAAAAATATGATGAGTTGCAhrVaProTrpValAsnAspSerMetGlnProGluTrpGlnAsnMetThrTrpGlnGlnTrpGluLysGlnIleAlaPheLeuGluAspAsnIleThrGluLeuLeuGluGlnAlaGlnIleGlnGlnGluLysAsnMetTrpTrpGluLeuG1					B10	12/17		
	-----G-----					B3	3/17		
	-----G-----					B1	1/17		
	-----G-----					B2	1/17		
5/89	GAAATTAATAGCTGGCATGTTTTGGCAATGGCTGATCTCAGCTCTGGCTAAATAATATCTATTATTAGGAATTATTAATAGACAGCAGAGTAATAGAGGTAAGAGATAGCTATCTATGTAGTACAAAGTTAATGAGACTTAGGAAGCGCnlysLeuAsnSerTrpAspValPheGlyAsnTrpLeuAspLeuThrSerTrpValLysTrpIleTyrIleGlyPheTyrIleValAlaGlyValIleValLeuArgIleAlaIleTyrValValGlnMetLeuMetArgLeuArgLysCly					B10	12/17		
	-----G-----					B3	3/17		
	-----G-----					B1	1/17		
	-----G-----					B2	1/17		

7312A env

M3 clones	
9/90	GACCTGCTCAAGGACCAACAAGAAATGTCGCGATCAGCCTCTGGGGACAACAAAACTCCAGCGAAGAGTCACTGCTATTGACAAAATACCTAAAGGACCAAGCGCCAACTAAATTCATCGGGATGCCCTTTACGCAAGCTGCCCACTA AspValValIysArgGlnGlnMetLeuArgLeuThrValTyrGlyThrIysAsnLeuGlnAlaArgValThrAlaTcGluLysTyrIleuLysAspGlnAlaGlnIleuAsnSerTyrGlyCysAlaProArgGlnValCysHisThrT D35 2/3
9/90	CTGTACCATGGTAAATGACAGCTTGACACCGATGATGGGACAACATGACCTGGCAACAATGGGAAAAACAAATCCGCTACCTGAGGCGCAATATCAGTCAAATCTTGAACAGCGCAAAATCCGACGAACAAACACATGTATGAATTACA IcValProTyrValAsnAspSerLeuThrProAspTyrAspAsnMetThrTyrGlnGlnTyrGlyLysGlnIleArgTyrLeuGlnAlaAsnIleSerGluSerLeuGluGlnAlaGlnIleGnglnGluLysAsnXetTyrGLeuGLeuG D21 1/3 def D21 1/1 cultured
9/90	AAATTAATATAGCTGGGATGTTTGGCACTGGTGTGATTTAGCCCTCGGGTCAAGTATATTCAGTATGGAGTCTATATAGTAGTAGGAATAGTACGCTCTPAGAATAATAATATGTAGTACAAATGATAGTACACTAGAAAGGT nLysLeuAsnSerTyrPaspValPheLysMetTyrPheAspLeuAlaSerTyrValIysTyrIleGlnnIyGlyValTyrIleValValGlyLeValAlaLsLeuArgCtltleTcTyrValValGlnMetIleGlyArgIleArgArgGly D35 2/3
9/90	D21 1/3 def D21 1/1 cultured

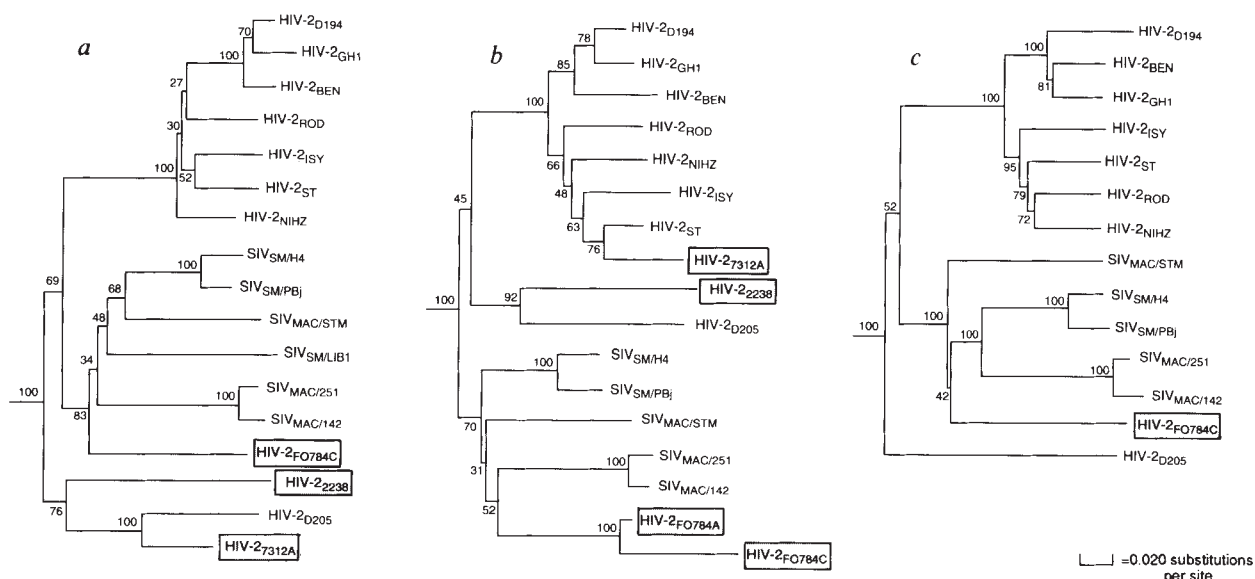


FIG. 3 Phylogenetic relationships among (a) *pol*-integrase, (b) *env*, and (c) *pol*-reverse transcriptase sequences from HIV-2_{F0784}, HIV-2₂₂₃₈, and HIV-2_{7312A} (boxed) and other HIV-2, SIV_{SM}, and SIV_{MAC} isolates¹². Horizontal branch lengths are to scale; vertical separation is for clarity only. Numbers at each node indicate the percentage of bootstrap samples (out of 5,000) in which the cluster to the right is supported. The trees are rooted using HIV-1, SIV_{AGM}, SIV_{CP2}, and SIV_{MND} sequences (not shown); branch lengths from the nearest node to the roots of the trees shown are 0.08, 0.11 and 0.11 substitutions per site for the *pol*-integrase, *env*, and *pol*-RT trees, respectively.

METHODS. Representative *pol*-integrase (F0784pol.C12: GenBank/EMBL accession number M87110; 2238pol.B7: M87138; 7312Apol.DS1: M87145), *env* (F0784env.A13: M87069; F0784env.C9: M87090; 2238env.B10: M87118; 7312Aenv.D35; M87142), and *pol*-RT (F0784rt.C2: M87111) sequences were analysed using the neighbour-joining method¹⁶ applied to a distance matrix of substitutions per site, corrected for multiple hits by the 2-parameter method²⁸, and implemented using the CLUSTALV package²⁹. Phylogenetic analyses were also performed by the maximum parsimony method¹⁵, implemented using the MULPARS option of PAUP³⁰ and the DNAPARS program of PHYLIP¹⁷ (which, with repeated randomized sequence input order, gave identical results). Neighbour-joining and maximum parsimony analyses produced virtually identical topologies which differed only in non-significant aspects of the relative branching order. For example, maximum parsimony analysis of the *pol*-integrase sequences yielded four

trees of length 1,422 (excluding gap mutations) compared to the neighbour-joining tree of length 1,423, and maximum parsimony analysis of reverse transcriptase sequences yielded one tree of length 3,781 compared with the neighbour-joining tree of length 3,782. In each case, the trees differed only in the relative branching order within the two major lineages (that is, prototypic HIV-2 and SIV/HIV-2_{F0784} subgroups). Analysis of *env* sequences by maximum parsimony yielded two trees of length 1,024 which differed from the neighbour-joining tree (length 1,032) by placing the HIV-2_{D205/2238} cluster outside the prototype HIV-2 and SIV/HIV-2_{F0784} lineages (neither analysis could definitively resolve this aspect of the branching order). Importantly, in none of the three genomic regions examined did the topological differences affect the positions of HIV-2_{F0784}, HIV-2₂₂₃₈ or HIV-2_{7312A}. The recombinant nature of HIV-2_{7312A} and the clustering of HIV-2_{F0784} with the SIV lineage were also confirmed by testing how many additional substitutions were required to (artificially) position HIV-2_{7312A} *pol* with HIV-2_{ST} (1,499 compared with 1,422), HIV-2_{7312A} *env* with HIV-2_{D205} (1,064 compared to 1,024), and HIV-2_{F0784} RT with the prototype HIV-2 lineage or HIV-2_{D205} (3,819 and 3,841 compared with 3,781). Finally, bootstrap analyses (200 replicates) of the maximum parsimony trees (using DNABOOT from the PHYLIP package¹⁷) gave repeatability values similar to those from the neighbour-joining analyses. A complete description of all sequences used for the phylogenetic studies, including alignments and accession numbers, and the results of the maximum parsimony analyses are available on request from the authors.

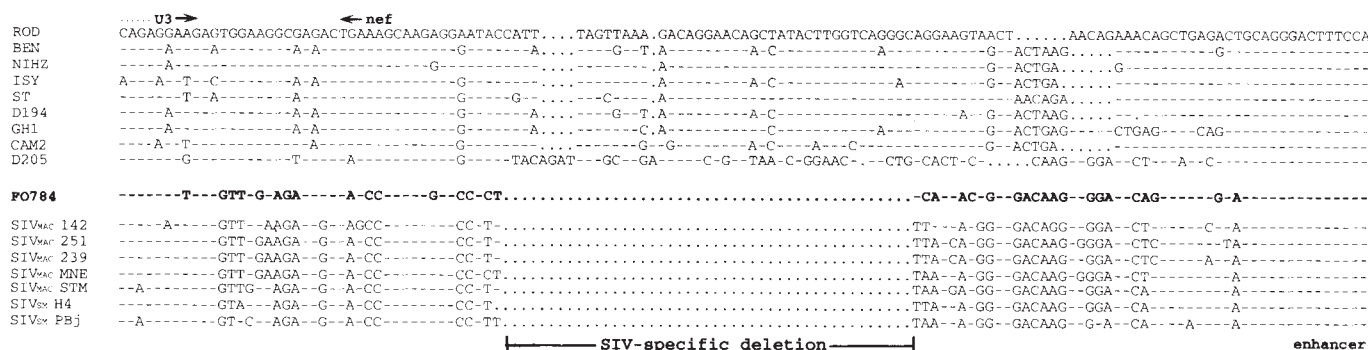


FIG. 4 Identification of an SIV_{SM}/SIV_{MAC} 'signature' sequence in HIV-2_{F0784}. HIV-2_{F0784} LTR sequences are aligned with prototype HIV-2 and SIV sequences in a region of the LTR encompassing a specific 40-44-bp insertion which distinguishes all previously reported HIV-2 viruses from SIV_{SM}/SIV_{MAC} viruses¹². Sequences are compared to HIV-2_{ROD} (ref. 1) as a reference sequence, with dashes indicating sequence identity and dots indicating gaps

introduced for optimal alignment. Positions of enhancer sequences and the *nef* termination codon are indicated. HIV-2_{F0784} (bold face), like viruses of monkey derivation, lacked the 40-44-bp insertion.

METHODS. The 717-bp *nef*/LTR fragment was amplified from uncultured F0784 PBMC DNA, cloned (F0784LTR.C5: M87115), and sequenced as described in Fig. 1.

F0784 contained multiply defective genomes and an inordinate number of G-to-A substitutions. Biased G-to-A hypermutation has previously been described for HIV-1^{14,25}, but not to the extent found in HIV-2_{F0784}. But in macaques experimentally infected with SIV_{SM}, extensive G-to-A hypermutation was found which correlated with reduced viral pathogenicity²⁶. Studies are presently underway to elucidate the molecular basis of G-to-A hypermutation and to determine whether, in the extreme case, it could result in attenuated or even abortive HIV-2 infection.

Finally, a recent suggestion²⁷ that SIV_{SM} may have been accidentally transmitted to man by inoculation with infected monkey blood probably cannot explain the diversity now recognized for HIV-2. Our results re-emphasize the need to target viruses from feral monkey populations and humans living in remote areas of Africa in a search for the origins of human immunodeficiency viruses and events leading to their recent epidemic spread. □

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A single point mutation is the cause of the Greek form of hereditary persistence of fetal haemoglobin

Meera Berry, Frank Grosveld & Niall Dillon

Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

IN normal humans the fetal stage-specific γ -globin genes are silenced after birth and not expressed in the adult. Exceptions are seen in cases of hereditary persistence of fetal haemoglobin (HPFH). These are clinically important because the elevated levels of γ -globin can alleviate β -thalassaemia and sickle cell anaemia. One class of mutations is associated with point mutations in the promoter of the γ -globin genes (non-deletion HPFH), whereas others seem to be caused by large deletions 3' to the γ -globin genes¹. To test whether the point mutation found in the Greek non-deletion HPFH^{2,3} (guanine to adenine at nucleotide position –117) is the cause of the raised γ -globin levels in the adult stage and is not just a linked polymorphism, we engineered this mutation into a γ -globin gene. When this gene was introduced into mice, the presence of the –117 mutation results in persistence of γ -globin expression at a high level and a concomitant decrease in β -globin expression in fetal and adult mice. We show that these changes correlate with the loss of binding of the transcription factor GATA1 to the γ -globin promoter, suggesting that it may act as a negative regulator of the γ -globin gene in adults.

Two globin minilocus constructs were injected into fertilized mouse eggs. The first construct contained a wild-type (wt) γ -globin gene flanked by the entire locus control region (LCR) and a β -globin gene⁴ (Fig. 1). The second construct was the same wild-type locus but with a single engineered point mutation at position –117 (G $\rightarrow$ A) in the promoter of the γ -globin gene. This mutation was verified by sequence analysis (not shown). The β -gene was included as a reference gene for quantitation and to allow rapid analysis of the construct without the need

to establish a large number of bred lines. When the wild-type $\gamma\beta$ minilocus was introduced into fertilized mouse eggs, five transgenic mice were obtained. Southern blots showed that two of the founders were mosaic (31 and 36) and that all contained the intact $\gamma\beta$ minilocus, albeit at different copy numbers (Table 1, and data not shown). S1 nuclease protection analysis showed that the γ -globin gene expression was suppressed in adult mice (Fig. 1a, b). In contrast, the human β -globin gene was expressed at this stage at levels comparable to those observed for the mouse β -maj-globin genes⁵ (Fig. 1b; Table 1). The suppression of the wild-type γ -globin gene is in agreement with results obtained when a minilocus containing only the γ -globin gene is introduced into mice⁴. Repeated phlebotomy increases the number of reticulocytes, but even under those conditions the γ -globin gene remains suppressed (Fig. 1b). When the –117 mutant $\gamma\beta$ minilocus was introduced into mice, nine transgenic mice were obtained and Southern blots showed that they contained intact miniloci at different copy numbers, although a number were mosaic (Table 1, and data not shown). S1 protection analysis showed a completely different result from that obtained with the wild-type $\gamma\beta$ minilocus. The –117 mutant γ -globin gene is now expressed at high levels in the adult stage in eight out of nine founder transgenic mice (Fig. 1a, c). Line 7, which does not express γ at the adult stage, was found to express the γ gene at the embryonic and early fetal stages (not shown). Analysis by polymerase chain reaction (PCR)⁶ of the γ -globin gene promoter in line 7 showed that it still contained the –117 mutation, thus ruling out a reversion of the mutation (not shown). The line 7 γ gene was not analysed for other mutations and we have therefore, as yet, no explanation for this exception. The expression level of the γ gene in the other HPFH mice varied considerably (Fig. 1; Table 1), which may be caused by the different arrangement of the transgene loci (P. Fraser and N.D., unpublished results; for example, the three lowest γ/β expressors all have a head-to-head integration) and the fact that the developmental regulation of the γ gene is very sensitive to position effects^{4,7,8}.

To determine whether expression of the γ -globin gene at the adult stage in the HPFH mice leads to a partial suppression of the linked human β -globin gene, all RNA samples of the wild type and HPFH $\gamma\beta$ minilocus-containing mice were compared

directly using probes of comparable specific activity for all the genes in the same experiment (Fig. 1, and data not shown). Quantitation of the signals (Table 1) suggests that the expression of the human β -globin gene in HPFH mice is reduced significantly per gene copy and that the total output per locus is slightly reduced in the adult bred mice (99 and 61) when compared with either the mouse β -maj-globin gene or the human β -globin gene in the wild-type $\gamma\beta$ mice. This provides further evidence that the γ gene competes with the β gene for the LCR⁹⁻¹¹ and correlates well with the lower levels of expression of the β -globin gene that is allelic with the HPFH gene in humans^{12,13}.

The effect of the HPFH mutation at earlier stages of development was also examined by analysing staged embryos from lines 99 and 61 (Fig. 2). In embryonic yolk sac the pattern of expression for both the wild-type and HPFH constructs was similar to that previously reported for LCR $\gamma\beta$ constructs, with γ expressed and β suppressed^{9,10}. In 13.5-day fetal liver there is a striking difference between the wild-type and HPFH constructs. The wild-type $\gamma\beta$ mice gave similar levels of γ and β expression, but in HPFH $\gamma\beta$ mice the expression of the human β gene is almost completely suppressed ($\gamma > 96\%$ of $\gamma + \beta$). Because this is significantly different from the $\gamma/\gamma + \beta$ ratio observed in adult animals (Table 1), it is further evidence that the balance of transcription factors is substantially different at the fetal liver stage than at the adult stage of mouse erythropoiesis⁴. Our data are different from the results of Morley *et*

*al.*¹⁴, who did not obtain expression of an HPFH gene in the adult mouse. Although we cannot explain this difference at present, it should be pointed out that their construct¹⁴ contained only site 2 and lacked the LCR elements that are most active in the adult (P. Fraser and F.G., unpublished results).

Our data show that a mutation in the distal CAAT box can override the adult suppression of the γ -globin gene. This could result from the loss of a bound suppressor or the gain of a bound activator. The 13-base-pair deletion¹⁵ that removes the distal CAAT box (Fig. 3) would favour the first possibility. Previous attempts to determine whether the -117 mutation affects the binding of transcription factors have been hampered by lack of good adult-stage human erythroid cell lines. Some differences have been reported in the binding of factors and CDP (ref. 16), NFE3 (ref. 17) and CP1 (refs 16, 18) have all been considered as candidates for a role in γ suppression through binding to this region (Fig. 3). Because the wild-type

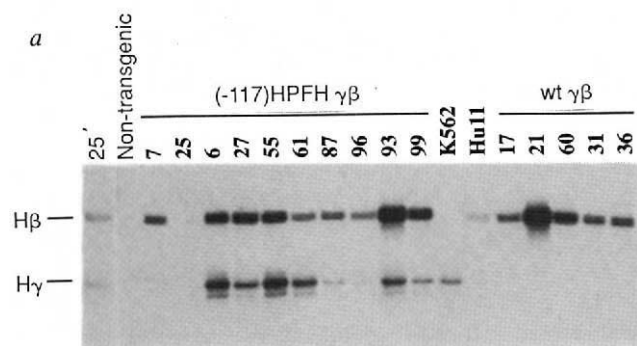
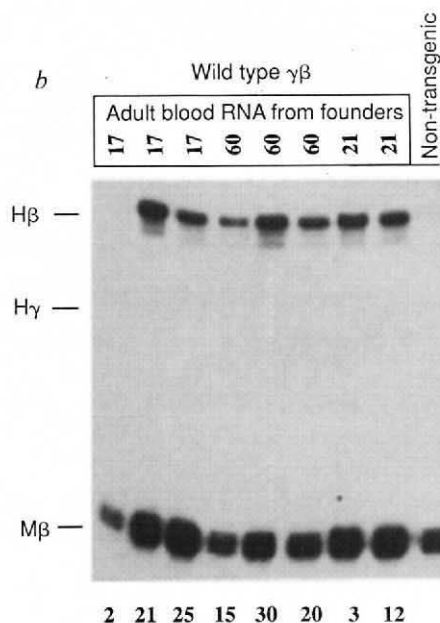
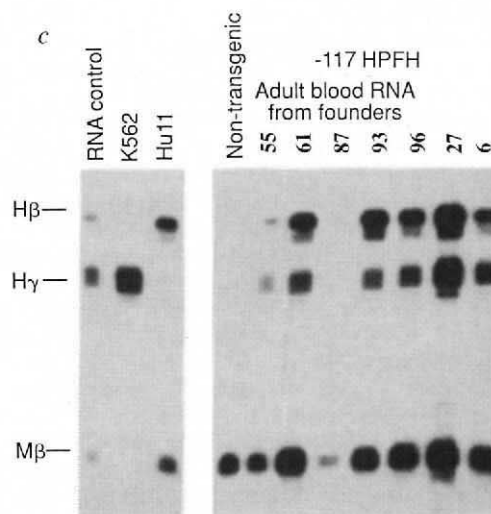


FIG. 1 Expression of wild-type (wt) $\gamma\beta$ and -117 HPFH $\gamma\beta$ minilocus constructs in transgenic mice. Schematic representation of the wild-type $\gamma\beta$ and Greek HPFH $\gamma\beta$ minilocus constructs are shown in *b* and *c*. The wild-type or Greek HPFH minilocus constructs were made by cloning a 4.98-kb *Bgl*II fragment containing the human β -globin gene into the LCR- γ -globin construct⁵. The Greek HPFH $\gamma\beta$ minilocus was made by replacing the wild-type promoter with the equivalent mutagenized promoter containing the G→A base change at position -117 upstream of the γ A CAP site. *a*, S1 nuclease analysis of all transgenic mice (numbered as in Table 1), using probes of equal specific activity for human β and γ RNA (H β , H γ). Controls were; γ -globin RNA from K562 cells; β -globin RNA from Hu11 cells. Line 99 was bred from founder 27 and has a single transgene integration site. Founder 27 had multiple integrations. Leftmost lane (25') shows a higher exposure for mouse 25. *b*, S1 nuclease analysis of three wild-type $\gamma\beta$ mice after phlebotomy. Probe specific activities (H γ /H β /M β) were 1/1/5. The reticulocyte count (%) is indicated below the lanes. *c*, S1 nuclease analysis of seven -117 HPFH $\gamma\beta$ mice. Probe specific activities (H γ /H β /M β) were 0.5/0.8/1. RNA controls were a mixture of γ and β RNA: γ RNA from K562 cells and β RNA from Hu11 cells.

METHODS. *Sal*I fragments containing either the wt $\gamma\beta$ minilocus or Greek HPFH $\gamma\beta$ minilocus were injected into mouse oocytes to generate transgenic mice. RNA was prepared from blood of adult transgenic mice and human γ , β and mouse β -maj globin RNA levels were compared by S1 nuclease protection analysis using a 5' end-labelled 525-bp *Acc*I fragment of the human β -globin gene, a 190-bp *Bst*NI fragment of the A γ gene and a 700-bp *Nco*I-*Hind*III fragment from the mouse β -maj gene⁴. The 5' end of human γ , β and mouse β -maj globin mRNA protect 140, 160 and 95 nucleotides of these probes respectively. All Southern blot and S1 nuclease protection signals were quantitated by scanning on a phosphorimager.



wt $\gamma\beta$ minilocus LCR γ β 40 kb



-117 $\gamma\beta$ minilocus LCR -117 γ β 40 kb

TABLE 1 Copy numbers of transgenic mice and relative expression of human and mouse globin gene

Mouse	Copy number	Human β /mouse β	Human $\gamma + \beta$ /mouse β	Human γ /human $\gamma + \beta$
HPFH				
6	3-4†	9	20	55
25	3	3	6	50
27	14†	6	10	40
61*	1	30	90	65
87	<1†	20	30	40
93	3	30	45	30‡
96	2	20	30	30‡
55	5	25	60	60
99*	3	40	60	30‡
7*	2	80	80	<1
Wild type				
17	<1†	>70	>70	<1
60	5-6	100	100	<1
21	2	100	100	<1
31	13†	5	5	<1
36	3†	10	10	<1

First column: mouse number; *, bred lines. These lines are the only lines guaranteed not to be mosaic. Line 99 was generated from founder 27 (see Fig. 1a). Second column: number of intactly integrated transgenic loci; †, mice mosaic for the transgene based on Southern blot analysis, not all mosaics can be detected this way. Third column: per cent human β -globin RNA of mouse β -globin RNA per gene copy. Fourth column: per cent total human globin RNA of mouse β -globin RNA per locus. Fifth column: per cent human γ -globin RNA of total human globin RNA in adult mice; ‡, head-to-head integration of injected fragments. Results are the average of three S1 nuclease protection experiments. All S1 nuclease protection and Southern blot signals were quantitated on a phosphorimager.

γ -globin transgene is expressed at the early fetal stage and suppressed at late stages in mice, we used nuclear protein extracts from embryonic yolk sac, fetal liver, a mouse erythroid leukaemia (MEL) cell line (adult stage) and from non-erythroid HeLa and F9 cells to investigate which protein factors are bound to the distal CAAT box region, and which of those would show a correlation with developmental stage-specific expression and the HPFH mutations. Oligonucleotides corresponding to the wild type and the -117 mutation were used in gel retardation experiments (Fig. 3, and data not shown). First the wild-type and -117 HPFH probes were used to determine the number of complexes at different developmental stages. Figure 3 shows that three prominent bands and several weak bands were formed at the different developmental stages. As shown previously, the slowest migrating complex corresponds to the CAAT box binding factor CP1 (ref. 18), which shows no increase in binding

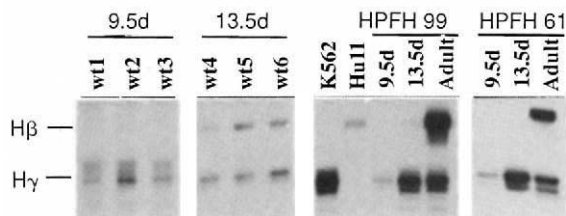


FIG. 2 S1 nuclease mapping analysis of minilocus wild-type $\gamma\beta$ and Greek HPFH $\gamma\beta$ mice during development. RNA was prepared from transgenic 9.5-day yolk sacs, 13.5-day fetal livers and from blood of adult mice. Yolk sacs and fetal livers containing minilocus wild-type $\gamma\beta$ (wt 1-6) were analysed directly after microinjection; yolk sac, fetal liver and adult blood containing the Greek HPFH $\gamma\beta$ construct were obtained by breeding lines 99 and 61. Control RNAs were from K562 and Hu11 cells. The specific activity of the γ and β probes was 1:1, except in panel HPFH 61, for which the ratio γ : β is 1:2.

during development and no significant difference in binding to the wild-type or -117 HPFH CAAT boxes. It therefore does not correlate with the developmental expression of the wild-type gene and the lack of suppression of the -117HPFH γ gene and is unlikely to be the factor providing stage specificity, although it may be part of a suppressor complex involving multiple factors. A fast-migrating band (NF-E6) also does not increase significantly during development. This band represents the principal complex formed when the β -globin CAAT box is used for binding *in vitro* (not shown). It is absent from HeLa and F9 cells and therefore appears to be an erythroid-specific protein complex. It does not correlate with changes in transcription of the wild-type gene and binding is not affected by the HPFH mutations. CDP also fails to show the change in levels during development (not shown) that would be expected of a suppressor. The only complex that does show correlation with the expression patterns of the wild-type and HPFH γ -globin genes is the complex migrating between CP1 and NF-E6, which is formed by GATA1 as shown by competition (although it self-competes much less effectively than a perfect GATA1 binding site) and antibody binding experiments (Fig. 3). As reported

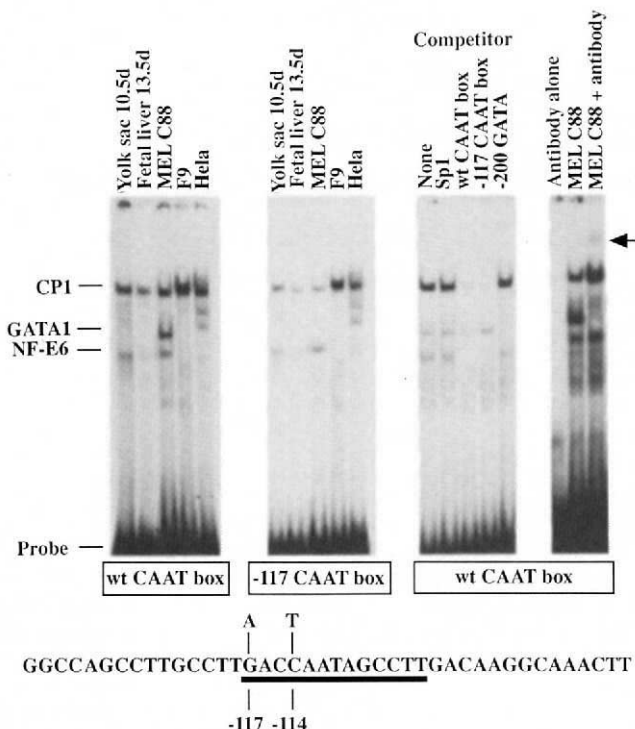


FIG. 3 Gel retardation analysis using wild-type (wt) and -117 γ -globin CAAT box. The γ -globin wild-type (left panel) or -117 G→A distal CAAT box (second panel) was subjected to gel retardation analysis using nuclear extracts prepared from embryonic yolk sac (10.5 d.p.c.), fetal liver (13.5 d.p.c.), adult mouse erythroleukaemic cell line (MEL, C88), non-erythroid uninduced F9 cells and HeLa cells. The third panel shows a competition assay using the wt CAAT box as the probe. The competitors were an Sp1 binding site, wt or -117 γ -globin CAAT box and a consensus GATA1 binding site from the human β -globin promoter²³. The right-hand panel shows a supershift with a GATA1 monoclonal antibody (arrowed). The first lane contains only the antibody, lane 2 only MEL extract, and lane 3 has both extract and antibody. The sequence of the CAAT box oligonucleotides is shown at the bottom. The numbers indicate the position relative to the γ globin CAP site. The solid bar indicates the 13-bp deletion¹⁵.
METHODS. Nuclear extracts from mouse tissues or cell lines were prepared as described²³⁻²⁵. Each gel retardation assay (10 μ l) contained ~5 μ g nuclear protein, 2 μ g poly(dI · dC) and 0.5 ng of 5' ³²P end-labelled double-stranded wild type or -117 HPFH oligonucleotides, and was done as described²³. Extracts used in each assay had similar levels of Sp1 transcription factor as judged by gel retardation using a CACC double-stranded oligonucleotide²⁵.

previously¹⁹, the relative amount of GATA1 binding activity increases during development. Figure 3 shows that this GATA1 binding is not observed with extracts from the yolk sac or fetal liver when the wild-type γ gene is still expressed (Fig. 2). In extracts from adult-stage cells, when the HPFH but not the wild-type gene is expressed, GATA1 binds to the wild-type but not to the HPFH CAAT box (Fig. 3). This suggests that GATA1, in contrast to its reported transactivation properties^{20,21}, may also function as a component of stage-specific suppression of the human γ -globin genes.

In conclusion, we have provided evidence that a single point mutation in the γ -globin promoter is the cause of the HPFH phenotype and that the suppression of the γ -globin gene in mice closely mimics the suppression observed in humans. The only qualitative difference that we have detected in mice is the absence of γ -globin reactivation in response to erythroid stress, which is observed in humans²². For therapy in β -globin-related haemoglobinopathies, a specific reactivation of the γ -globin genes would be more useful than the stress-related reactivation. Our results showing that an HPFH mutation is functional in mice and that this correlates with loss of binding of a transcription factor, indicates that procedures to achieve specific γ -globin reactivation could be developed in the absence of the stress-related pathway using our mouse model system. □

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Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy

M. Sendtner*, H. Schmalbruch†, K. A. Stöckli*, P. Carroll*, G. W. Kreutzberg‡ & H. Thoenen*

Departments of *Neurochemistry and †Neuromorphology, Max-Planck-Institute for Psychiatry, D-8033 Martinsried, Germany
‡Institute of Neurophysiology, Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark

CILIARY neurotrophic factor (CNTF) supports the survival of embryonic motor neurons *in vitro*^{1,2} and *in vivo*³, and prevents lesion-mediated degeneration of rat motor neurons during early post-natal stages⁴. Here we report that CNTF greatly reduces all the functional and morphological changes in *pmn/pm* mice⁵, an autosomal recessive mutant leading to progressive caudo-cranial motor neuron degeneration. The first manifestations of progressive motor neuronopathy in homozygous *pmn/pm* mice become apparent in the hind limbs at the end of the third post-natal week, and all the mice die up to 6 or 7 weeks after birth from respiratory paralysis. Treatment with CNTF prolongs survival and greatly improves motor function of these mice. Moreover, morphological manifestations, such as loss of motor axons in the phrenic nerve and degeneration of facial motor neurons, were greatly reduced by CNTF, although the treatment did not start until the first symptoms of the disease had already become apparent and substantial degenerative changes were already present. The protective and restorative effects of CNTF in this mouse mutant give new perspectives for the treatment of human degenerative motor neuron diseases with CNTF.

We have evaluated the effects of CNTF in the *pmn/pm* mouse, which is an animal model for human spinal motor neuron disease⁵. In contrast to two other mouse mutants, *wobbler*^{6–8} and *mda*^{9,10}, the manifestations of motor neuron degeneration in *pmn/pm* mice appear earlier and progress more rapidly. In 4-week-old *pmn/pm* mice, the number of axons of the phrenic nerve is already highly reduced, indicating that at this time the

TABLE 1 Effect of CNTF treatment on the number of facial motor neurons and phrenic nerve axons in *pmn/pm* mice

	Number of facial motor neurons	Number of phrenic nerve axons
<i>pmn</i> mice (40–50 days old) (<i>n</i> =6)	1,881 ± 199*	87 ± 4*
CNTF-treated <i>pmn</i> mice (40–48 days old) (<i>n</i> =7)	2,679 ± 108*	144 ± 22*
Healthy control mice (littermates) (<i>n</i> =5)	3,108 ± 153	ND

The brain stem of mice perfused with 4% formaldehyde was embedded in paraffin, serial sections 7- μ m thick were stained with cresyl violet, and the nucleoli of facial motor neurons were counted in every fifth section on both sides as previously described⁴. Counts were not corrected for split nucleoli^{4,15}. The mean of the counts on both sides was used for each animal. Phrenic nerves were prepared after perfusion of the animals with 4% formalin. Nerves were postfixed in 4% formalin, dehydrated, then 5- μ m transverse sections made and stained according to ref. 16. Myelinated axons were counted from photographs taken from nerve sections under the light microscope. Data shown are means $\pm$ s.e.m. for each group. ND, not determined.

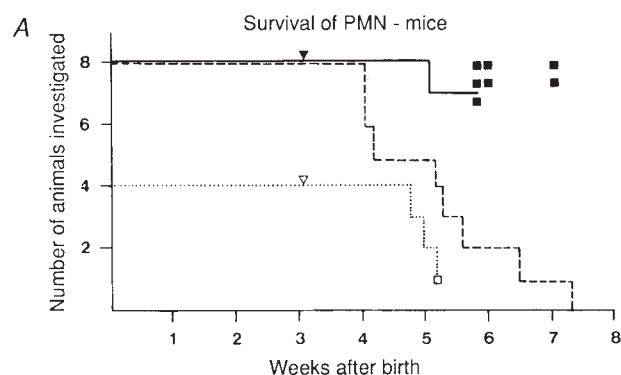
* Statistical significance was tested by Student's *t*-test, *P* < 0.0005.

disease has already reached an advanced stage. The motor neurons of *pmn/pm* mice first undergo a reduction in cell size, then chromatolysis and finally cell death, similar to the pathological changes seen in many cases of human motor neuron diseases¹¹. The gene defect responsible for the motor neuron changes in *pmn/pm* mice is still unknown. But an insufficient or defective expression of CNTF does not seem to be responsible for the degenerative changes. Northern blots of sciatic nerve reveal CNTF transcripts with similar size and intensity to those of the healthy controls. Western blots from sciatic and facial nerve extract are indistinguishable from those of controls and the same extracts contain comparable CNTF biological activity, as determined in the embryonic chick ciliary neuron survival assay (data not shown).

Because intravenously injected CNTF has a half-life of only a few minutes (F. Dittich and M.S., unpublished results) and

FIG. 1 A, Effect of CNTF on the survival of *pmn/pm*n mice. Survival of eight untreated *pmn/pm*n mice (dashed-line); eight *pmn/pm*n mice injected at post-natal day 20 or 21 with 5×10^7 CNTF-secreting D3 cells (solid line); and four *pmn/pm*n mice injected with 5×10^7 untransfected D3 cells (dotted line) are shown. Triangles, time of cell injection; squares, time of death. B, *Pmn/pm*n mice of two independent experiments at 35 (1 and 2) and 40 (3 and 4) days old. a, c, CNTF-treated *pmn/pm*n mice; b, d, untreated *pmn/pm*n litter mates.

*pmn/pm*n mice do not tolerate repetitive daily injections of CNTF, and because the available implantable infusion pumps are too large for 3–4 week-old mice, we established a stable cell line by transfection of mouse D3 cells¹² with a leader sequence/CNTF genomic DNA construct (Fig. 2a). CNTF is synthesized and released by these cells in high quantities. The effect of intraperitoneal injection of 5×10^7 CNTF-secreting D3 cells was compared with untreated controls from the same litters and with animals injected with untransfected D3 stem cells between post-natal day 20 and 21 (Fig. 1). Because homozygous *pmn/pm*n mice cannot be recognized before the onset of first paralytic symptoms, an earlier start of the treatment was precluded. Of four mice injected with untransfected D3 cells, three mice died between postnatal day 34 and 36. In comparison, five out of eight untreated *pmn/pm*n mice and seven out of eight



First symptoms : weakness of hindlimbs

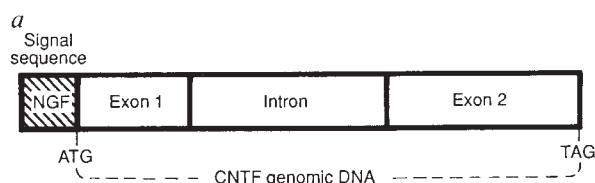
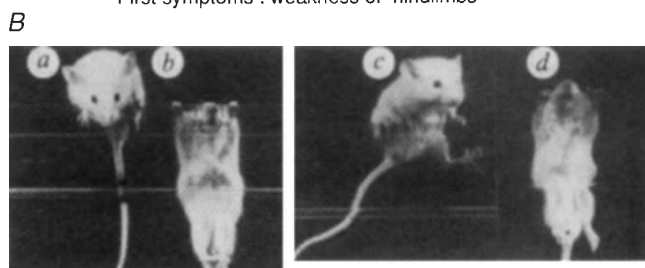
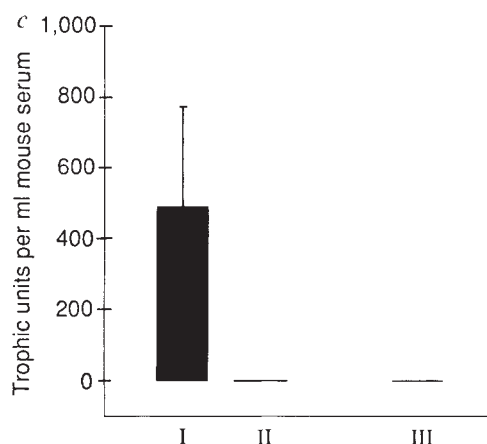
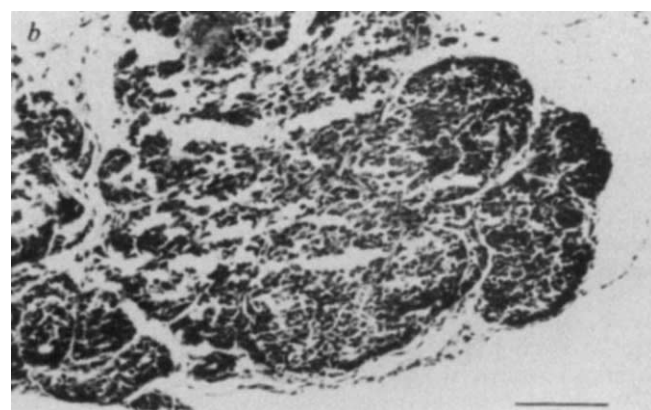


FIG. 2 a, Map of the construct used for stable transfection of mouse D3 stem cells: a fragment containing mouse CNTF genomic DNA, which includes the whole coding regions of exon I and II and a roughly 1,000-base pair (bp) intron, was cloned 3' of a complementary DNA fragment coding for the first 20 amino acids of mouse prepro-nerve growth factor (starting from methionine -121, which has been suggested as the translation initiation site for human and mouse NGF¹⁷), which includes the entire signal peptide sequence¹⁸. This construct was cloned into the pRc/CMV expression vector (In VitroGen). After linearization with *PvuI*, 25 μ g plasmid DNA were used for transfection of 5×10^6 mouse D3 embryonic stem cells by electroporation. Clones were selected by G 418 treatment¹² for neomycin resistance and analysed for CNTF expression. In contrast to cells transfected with native CNTF, which do not release CNTF^{19,20}, more than 70% of the total ciliary neuronal survival activity was released from these cells into the culture medium after 40 h in culture. b, Morphology of the intraperitoneal tumours that had formed 3 weeks after intraperitoneal injection of 5×10^7 D3 cells in a *pmn/pm*n mouse at postnatal day 21. Paraffin sections were prepared and stained with haematoxylin-eosin. Scale bar, 100 μ m. c, CNTF biological activity in: I, Sera from *pmn/pm*n mice after intraperitoneal injection of transfected CNTF-secreting D3 cells. The survival of isolated chick embryonic day-8 ciliary neurons was determined¹³ after addition of sera from *pmn/pm*n mice at dilutions from 1:1,000 to 1:100. One trophic unit is defined here as the volume of serum per ml of medium that supports half-maximal survival of the cultured neurons. II, A neutralizing antiserum against rat CNTF²¹ (prepared by repeated immunization of a rabbit with rat CNTF) was used as a control for the specificity of the bioassay for CNTF. This antiserum (5 μ l ml⁻¹ medium), which completely abolished the survival effect of 1 ng CNTF in cultures of ciliary neurons, inhibited the survival activity observed with the sera shown in I. III, Sera from four untreated *pmn/pm*n mice and from one *pmn/pm*n mouse treated with untransfected D3 cells were also tested and were inactive at the same concentrations (dilution 1:1,000 to 1:100 in the culture medium).



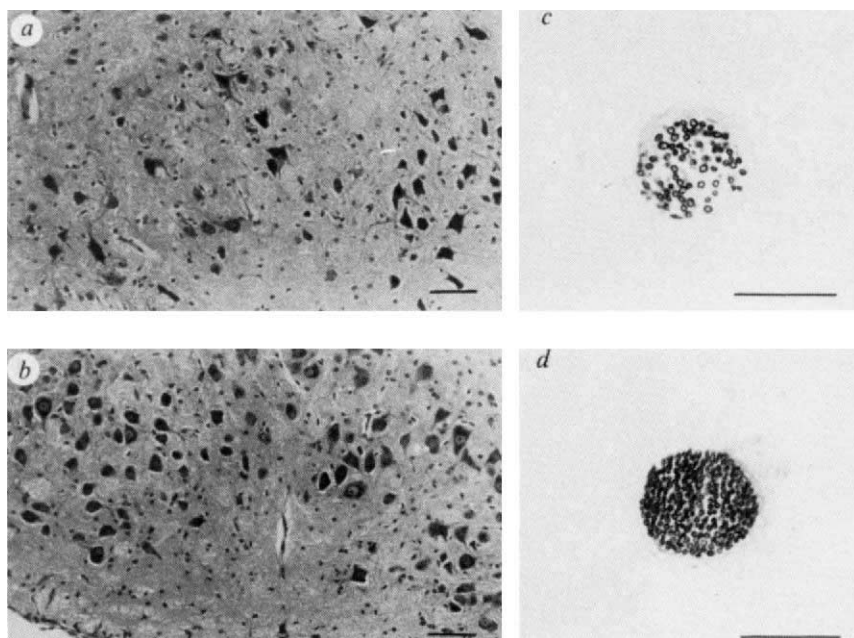


FIG. 3 Morphology of the facial nucleus (*a, b*) and phrenic nerve (*c, d*) of untreated *pmn/pmn* mice (*a, c*) and *pmn/pmn* mice injected with CNTF-secreting D3 cells (*b, d*). *a, b*, The motor neurons are more numerous, larger and vary less in diameter in the CNTF-treated mouse (*b*) than in the untreated mouse (*a*). Chromatolysis tends to be less pronounced after CNTF-treatment. *c, d*, Distal phrenic nerves of an untreated *pmn/pmn* mouse at day 43 and of a CNTF-treated *pmn/pmn* mouse at day 48. The nerve of the CNTF-treated animal (*d*) appears to be much better preserved and contains more myelinated axons than that of the untreated animal (*c*). Scale bars, 100 μm (*a, b*), or 50 μm (*c, d*).

CNTF-treated *pmn/pmn* mice were still alive at postnatal day 36. The fact that the intraperitoneal injection of untransfected D3 stem cells and the intraperitoneal growth of teratoma-like tissue (Fig. 2*b*) even increased the death rate in *pmn/pmn* mice is a further demonstration that these animals are very sensitive to any kind of surgical intervention.

Of eight CNTF-treated mice analysed, seven were still alive at postnatal day 40 when 50% of untreated *pmn/pmn* mice had already died (Fig. 1*A*). Moreover, these mice were capable of a better motor performance than the untreated *pmn/pmn* mice. In contrast to the latter, they could still climb onto a ruler when allowed to grip it with their forepaws (Fig. 1*B*).

CNTF-treated and non-treated mice were killed between postnatal day 40 and 48. After establishing deep ether anaesthesia, blood was taken by cardiac puncture and the mice were perfused with 4% formaldehyde. Brain stem, phrenic nerve and tumour tissue formed in the peritoneal cavity of D3 cell-treated animals were prepared for morphological analysis. Sera were tested for CNTF activity (Fig. 2*c*)^{13,14} and significant levels of ciliary neuronal survival activity (492 ± 282 trophic units ml^{-1} serum) were detected in the treated animals. Sera from untreated *pmn/pmn* mice did not show noticeable survival activity.

To determine motor neuron cell numbers we chose the facial nucleus because of the absence of interneurons in this well delineated structure. Interneurons present in the ventral horn of the spinal cord could erroneously be counted as atrophic

motor neurons. In comparison to untreated controls, the number of motor neurons in the facial nucleus was greatly decreased in *pmn/pmn* mice and the treatment with CNTF exhibited a large protective effect (Table 1; and Fig. 3*a, b*). CNTF supports the survival of single motor neurons in cell culture¹⁴, suggesting that the protective and restorative effects of CNTF in *pmn/pmn* mice are mediated directly on motor neurons rather than by skeletal muscle. Moreover, the number of axons in the phrenic nerve was significantly increased by CNTF treatment, that is 87 ± 4 axons in untreated and 144 ± 22 in CNTF-treated *pmn/pmn* mice (Table 1; and Fig. 3*c, d*).

The data presented indicate that CNTF efficiently rescues motor neurons from degeneration in *pmn/pmn* mice. Moreover, the large loss of axons in the phrenic nerve, presumably representing the cause of death in *pmn/pmn* mice, can be significantly reduced by CNTF treatment. Because initial symptoms cannot be recognized in *pmn/pmn* mice before the third postnatal week, it is to be expected that at this time the disease has already reached a relatively advanced stage, so that treatment can only begin when the degeneration of motor neurons has already started. The number of phrenic nerve fibres is already reduced to 30% in 28-day-old *pmn/pmn* mice⁵. The beneficial effects of CNTF in advanced stages of motor neuron degeneration in *pmn/pmn* mice indicates that treatment of human degenerative motor neuron diseases may eventually be possible. □

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CD21 is a ligand for CD23 and regulates IgE production

Jean-Pierre Aubry, Sibylle Pochon, Pierre Graber, Kathrin U. Jansen & Jean-Yves Bonnefoy*

Glaxo Institute for Molecular Biology, 14 chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

*To whom correspondence should be addressed

THE molecule CD23, a low-affinity receptor for IgE ($Fc\epsilon R_2$)^{1,2}, is a type II transmembrane molecule expressed on many haemopoietic cell types^{3,4}. CD23 has pleiotropic roles in the control of lymphocyte behaviour⁵⁻⁹, suggesting that CD23 may interact with another ligand in addition to IgE. To identify such a CD23 ligand, we expressed and purified full-length recombinant CD23, incorporated it into fluorescent liposomes and used these as a probe. We report here that fluorescent liposomes carrying CD23 interact specifically with the cell-surface protein CD21, identified as the receptor for Epstein-Barr virus and the complement receptor-2 on B cells, some T cells and follicular dendritic cells. In addition, fluorescent CD23-liposomes were shown to bind to hamster kidney cells (BHK-21) transfected with CD21 complementary DNA. The interaction between fluorescent CD23-liposomes and B cells or CD21-transfected BHK-21 cells was specifically inhibited by anti-CD21 and anti-CD23 monoclonal antibodies. Western blotting analysis revealed that ¹⁴C-labelled liposomes carrying CD23, in contrast to anti-CD21 antibodies, reacted with a subtype of CD21 molecules. Triggering of CD21 either with an anti-CD21 antibody or with recombinant soluble CD23 was shown to increase specifically interleukin-4-induced IgE production from blood mononuclear cells. These results demonstrate that the cell-surface protein CD21 is a ligand for CD23 and that the pairing of these molecules may participate in the control of IgE production.

Communication between different elements of the immune system is mediated both through direct cell-cell interactions and by soluble factors. B lymphocytes can be activated by several pathways, one of which is stimulated by monoclonal antibodies against CD23 (ref. 10). Recent reports have demonstrated the role of CD23 in B-cell growth^{5,6}, germinal centre B-cell survival⁷, prothymocyte maturation⁸, myeloid precursor proliferation⁹ and antigen presentation¹¹ by association with major histocompati-

bility complex (MHC) class II antigens¹². These findings led us to investigate whether CD23, previously defined as a low-affinity receptor for IgE^{1,2}, also interacts with a ligand other than IgE. Recombinant full-length CD23 was therefore expressed in insect cells using the baculovirus expression system⁶, purified to homogeneity, and incorporated into fluorescent liposomes for use as a tool to screen different human haemopoietic cell lineages for a novel ligand for CD23.

The human myeloma cell line RPMI 8226 showed the strongest binding of the fluorescent liposomes containing recombinant CD23 (Fig. 1). Recombinant CD23-transfected COS cells also interact specifically with RPMI 8226 cells by forming conjugates¹³. The binding of CD23-liposomes to RPMI 8226 was specifically inhibited by preincubating the CD23-liposomes with an excess of an anti-CD23 antibody (monoclonal antibody 25) (Fig. 1d), but not with a class-matched control antibody (data not shown).

To identify the ligand expressed on RPMI 8226 cells that interacts with CD23, we investigated whether any of a panel of 129 anti-B cell antibodies (coded B1 to B129 (ref. 14)) could block this interaction. Five antibodies inhibited the binding of CD23-liposomes to RPMI 8226 cells (Fig. 1a, b; code numbers B3: BU-32; B28: BU-33; B40: BU-34; B91: HB5; B93: BU-42), all of which were anti-CD21 antibodies. A control antibody that stained RPMI 8226 cells, the anti-CD38 antibody (code number B95: HB7), was unable to inhibit binding to CD23-liposomes (Fig. 1c). Two other anti-CD21 antibodies, coded B46 (B2) and B98 (B-Ly4), also failed to inhibit binding to CD23-liposomes, suggesting that they recognized a set of epitopes not involved in the interaction with CD23.

Further evidence of the specificity of the CD23-CD21 interaction was obtained by the binding of CD23-liposomes to non-lymphoid cells transfected with human CD21 cDNA. CD23-liposomes recognize recombinant CD21 expressed transiently on hamster kidney cells (BHK-21) (Fig. 2). The binding of CD23-liposomes to the CD21 transfectants could be competed by anti-CD21 antibodies (BU-33) (Fig. 2d), BU-32 and BU-34 (not shown) and inhibited by preincubation of the CD23-liposomes with an anti-CD23 antibody (monoclonal antibody 25) (Fig. 2e). In contrast, mock-transfected BHK-21 cells did not react with either anti-CD21 antibodies or CD23-liposomes (Fig. 2a, c). CD23-liposome binding was also observed on CD21-selected mouse fibroblast L-cells that had been

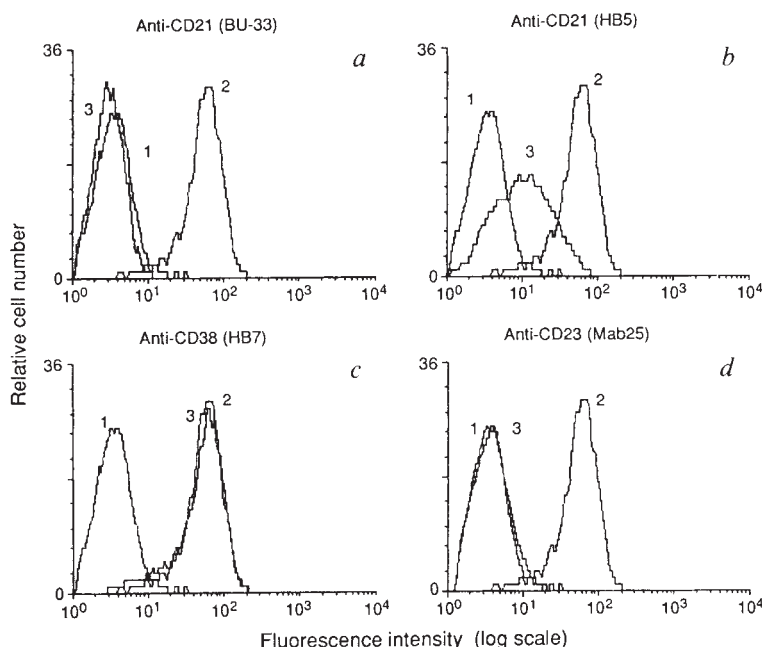
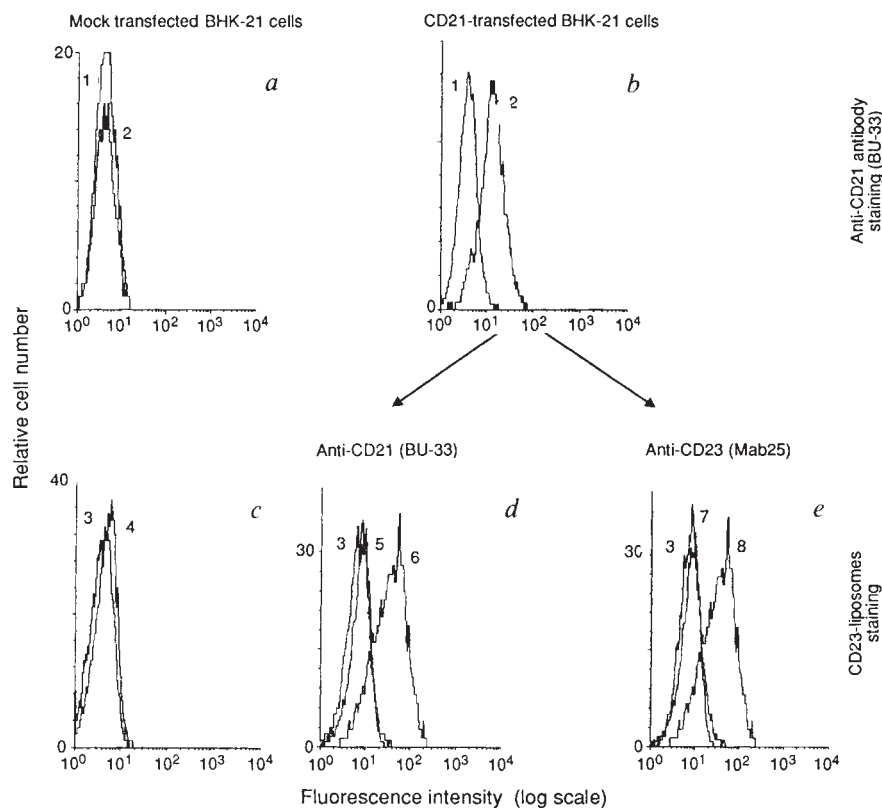


FIG. 1 Inhibition of CD23-fluorescent liposome binding by anti-CD21 antibodies as detected by flow cytometry. The recombinant full-length CD23 was expressed in insect cells using the baculovirus expression system⁶ and purified as described elsewhere¹³. Synthetic phospholipids were mixed with the fluorescent dye DiO₁₈ and dialysed against HEPES buffer together with purified recombinant CD23 or glycoprotein A (0.2 μ M each) (Calbiochem, La Jolla) as control protein (referred to as control-liposomes)¹³. RPMI 8226 cells were incubated with control liposomes (histogram 1), CD23 liposomes (histogram 2) in 0.5% BSA, 0.1% azide, 2 mM CaCl₂, 140 mM NaCl, 20 mM HEPES, pH 7 (referred to as HEPES buffer), or with either anti-CD21 antibodies (a and b) or control antibody (c), in the form of ascites¹⁴ diluted 1/25 in 0.5% BSA, 0.1% azide, PBS, pH 7 (referred to as PBS buffer) (specificity and clone designation are indicated above the histograms) followed by CD23-liposomes (histogram 3) for 1 h at 4 °C in HEPES buffer. d, CD23 binding specificity was obtained by preincubation of CD23-liposomes with anti-CD23 antibody for 30 min at 4 °C before the addition of RPMI 8226 cells. After two washes in HEPES buffer, 5,000 cells were analysed for each sample on a FACStar Plus (Becton-Dickinson, Erembodegem, Belgium) equipped with an argon laser tuned at 488 nm.

FIG. 2 Inhibition of fluorescent CD23-liposome binding to BHK-21 cells transfected with human CD21 cDNA by anti-CD21 and by anti-CD23 antibodies. The CD21 cDNA (from A. Calender) was obtained as an *EagI*-*XbaI* fragment which was treated with DNA polymerase I (Klenow fragment). After addition of *Bst*XI linkers, the fragment was subcloned into the *Bst*XI site of expression vector pCDM8 (Invitrogen). The resulting plasmid pCDM8-CD21.1 was used to transiently transfect BHK-21 cells (ATCC) using DEAE dextran as previously described³⁶. Flow cytometry profiles of mock transfected (*a* and *c*) and CD21-transfected BHK-21 cells (*b*, *d* and *e*) are shown. Cells were incubated for 30 min at 4 °C with control IgG₁ (histogram 1) or anti-CD21 antibody (histogram 2) at 10 µg ml⁻¹ in PBS buffer. After two washes in PBS buffer, antibodies were revealed by a second incubation with fluorescein-labelled sheep anti-mouse antibodies (Bioart, Meudon, France). Cells were incubated with control liposomes (histogram 3) or with CD23-liposomes (histogram 4) 1 h at 4 °C in HEPES buffer. CD21 binding specificity was evaluated by competition between CD23-liposomes and anti-CD21 antibody (BU-33) (histogram 5) or IgG₁ (histogram 6) at 10 µg ml⁻¹ in HEPES buffer. CD23 binding specificity was obtained by preincubation of CD23 liposomes with anti-CD23 antibody (Mab25) (histogram 7) or with control IgG₁ (histogram 8) at 10 µg ml⁻¹ in HEPES buffer. After two washes in HEPES buffer, cells were analysed by flow cytometry.



transfected with genomic DNA from Raji cells¹⁵ (data not shown). Taken together, these data demonstrate that CD21 is a novel ligand for CD23.

Interestingly, there was not a strict correlation between the level of CD21 expression detected by antibody staining on a cell and the capacity of this cell to bind CD23-liposomes. Western-blotting analysis of the CD23-binding cell line RPMI 8226 with anti-CD21 antibodies or with ¹⁴C-labelled liposomes carrying CD23 revealed that among three immunoreactive proteins, only one reacted with the ¹⁴C-labelled CD23-liposomes (Fig. 3). Because CD23 is a member of the C-type lectin family of adhesion molecules¹⁶ and because glycan structures are involved in the CD23 interaction with its ligand¹³, it is likely

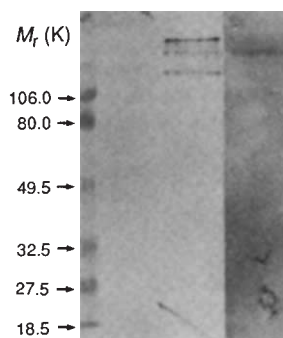


FIG. 3 CD23 interacts with a subtype of CD21 molecules. Membrane fractions of CD23-binding RPMI 8226 cells were separated on SDS-PAGE gels and transferred onto nitrocellulose. M_r markers are shown on the left. Filters were incubated with either a mixture of anti-CD21 antibodies (BU-33, B2 and HB5) then with horseradish peroxidase-conjugated goat anti-mouse antibody (Kpl; Gaithersburg, Massachusetts) followed by aminoethyl carbazole (lane second from right), or with ¹⁴C-labelled CD23-liposomes, followed by autoradiography (right lane). ¹⁴C-labelled CD23-liposomes were prepared by dialysing ¹⁴C-labelled phospholipids (L-3-phosphatidylcholine, 1-palmitoyl-2-(¹⁴C)-oleoyl, Amersham) against HEPES buffer together with purified recombinant CD23.

that CD23 binds to a glycomoiety on a subtype of CD21 which may not be present on all CD21 molecules.

A number of pieces of evidence support the idea that the CD21-CD23 interaction may play an important role in controlling lymphocyte responses. Certain anti-CD23 antibodies block the differentiation of B cells into IgE-secreting cells in response to interleukin-4 (IL-4)^{17,18}. We have suggested that the inhibitory effect of anti-CD23 antibodies on IgE synthesis could be due, at least partly, to the prevention of the CD4-positive T-cell-B-cell interaction (J. Shields *et al.*, manuscript in preparation), a process needed for IgE production¹⁹. On the basis of the finding reported here that CD21 is a ligand for CD23 and that CD21 is expressed on CD4-positive T cells²⁰, we investigated the effect of anti-CD21 antibody on T-cell dependent, IL-4-induced IgE production. An anti-CD21 antibody, BU-33, was shown to increase IgE production in an isotype-specific manner, whereas levels of IgG (Fig. 4), IgM and IgA were unaffected (data not shown). Conversely, an anti-CD23 antibody inhibited IgE production in an isotype-restricted manner (Fig. 4, and refs 17, 18). In addition, recombinant soluble CD23 expressed in COS cells in a form of a fusion protein with the extracellular domain of CD5 was also shown to enhance specifically IL-4-induced IgE production (Fig. 4, and ref. 21). Therefore triggering of CD21 with anti-CD21 antibody or with CD23 leads to an increase of IgE synthesis.

Human CD21 is a phosphoprotein of M_r 135-170K and is found on B lymphocytes²², some T lymphocytes²⁰, follicular dendritic cells²³ and pharyngeal epithelial cells²⁴. It is the receptor for the C3dg and iC3b proteins of the complement system²⁵ as well as for the gp350/220 envelope glycoprotein of Epstein-Barr virus (EBV)^{26,27} and also a receptor for interferon-α²⁸. Thus, the CD23-CD21 interaction may play a role in inducing cell proliferation. CD21 has been implicated in B-cell activation on triggering by EBV, C3d fragments or anti-CD21 antibodies^{27,29-32}. Consistent with the data presented here, different forms of soluble CD23 have been reported to activate human B cells⁵⁻⁷. Antibodies specific for human CD23 induce B-cell proliferation¹⁰ and increase intracellular Ca²⁺ concentration³³.

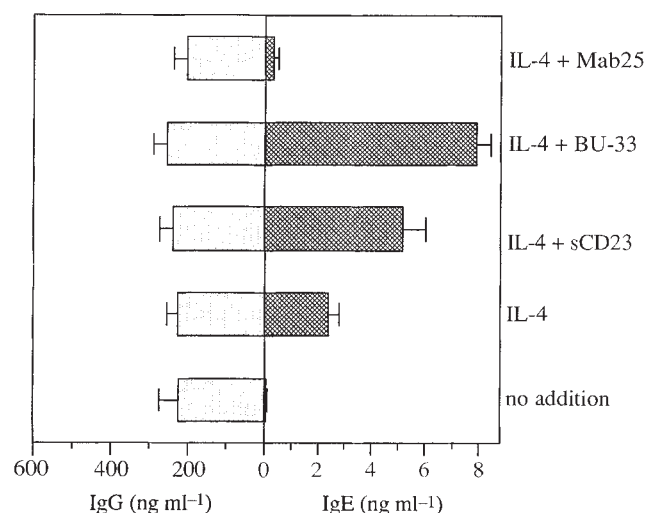


FIG. 4 Engagement of CD21 by an anti-CD21 antibody and by recombinant soluble CD23 increases IgE production. Peripheral blood mononuclear cells were incubated for 14 days alone or with 200 U ml⁻¹ recombinant IL-4 (Amersham). Purified antibodies to CD21 (BU-33) or to CD23 (Mab25) (both at 10 µg ml⁻¹) were used in the presence of IL-4. A CD23-CD5 fusion protein was obtained by joining an extracellular portion of the CD5 antigen (amino acids 1–344) to the 29K soluble CD23 (amino acids 119–321). The construct was made by cutting the CD5 cDNA (from I. Stamenkovic) with *HindIII* and *BamHI*, and cutting the CD23 cDNA (from H. Kikutani) with *BglII* and *PstI*. The relevant fragments were isolated by agarose gel electrophoresis and ligated into the COS cell expression vector pCDM8 which had been treated with *HindIII* and *PstI*. The resulting vector was transfected into COS-M6 cells as described³⁶. Three days after the transfection, the COS cell supernatant was collected and the amount of the fusion protein was determined by a CD23-specific ELISA³⁷. The CD23-CD5 fusion protein was used at 10 ng ml⁻¹ in the presence of IL-4, BU-33, Mab25 and the CD23-CD5 fusion protein had no effect on IgE synthesis in the absence of IL-4 (data not shown).

Moreover, EBV-B cell lines and nasopharyngeal carcinoma cells^{24,34} express both CD23 and its ligand CD21, and therefore the CD23-CD21 interaction may well contribute to auto-stimulatory growth of these transformed cells.

CD21 expressed on the surface of the follicular dendritic cells²³ may be involved in the trapping and survival⁷ of CD23-positive germinal centre B cells and in the presentation of the antigen to the B cell³⁵. We have shown that CD21 is a functional ligand for CD23 and suggest that the physical interaction between these two receptors is likely to have a major role in controlling a number of important aspects of the immune response. □

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Evidence for a viral superantigen in humans

Monique Lafon*, Mireille Lafage*,
Amalia Martinez-Arends*, Rafaël Ramirez†,
Françoise Vuillier‡, Dominique Charron‡,
Vincent Lotteau† & Daniel Scott-Algara‡

*Unité de la Rage, †Unité d'Immunohématologie et d'Immunopathologie, Institut Pasteur, 25–28 rue du Docteur Roux, 75724 Paris cedex 15, France

‡Laboratoire d'Immunogénétique, Institut Biomédical des Cordeliers, 15 rue de l'Ecole de Médecine, 75006 Paris, France

SUPERANTIGENS^{1–4} bind class II major histocompatibility proteins^{5,6} and stimulate powerful proliferative responses of T lymphocytes bearing particular Vβ sequences as part of their αβ antigen receptor⁷. Exogenous bacterial superantigens are responsible for food poisoning and toxic shock syndrome. Murine virus-encoded self-superantigens induce clonal deletion of T lymphocytes. Although superantigen-like properties have been suggested for human immunodeficiency virus-1, no viral superantigen has been identified in humans⁸. Here we report that the nucleocapsid of the rabies virus is an exogenous superantigen specific for Vβ8 human T lymphocytes which binds to HLA class II α-chains.

The nucleocapsid of the rabies virus is a potent activator for peripheral blood lymphocytes (PBL) of human rabies vaccinees⁹. The nucleocapsid is composed of a negative-strand RNA coated with three internal proteins: N, NS and L proteins. The nucleocapsid was purified from hamster rabies-infected cells¹⁰ and its major constituent, the N-protein, was produced from insect cells infected with N-protein-recombinant baculovirus¹¹. The following data were first obtained with nucleocapsid and confirmed with purified N-protein. T and B lymphocytes from unprimed (non-vaccinated) and primed (vaccinated) donors were incubated with purified nucleocapsid and N-protein and the proliferative response was compared to T-cell proliferations induced by tetanic toxoid (TT, a conventional antigen) and toxic shock syndrome toxin (TSST-1, a bacterial superantigen) (Table 1). Nucleocapsid and N-protein could not stimulate purified T or B cells alone, indicating that they are not classical mitogens. A proliferative response was only observed when both T and B cells are present. T cells responded to nucleocapsid, N-protein and TSST-1 presented by fixed B cells but not to TT under the same conditions. In contrast to conventional antigen, which must be processed to initiate a T-cell response, native N-protein and nucleocapsid can be presented to T cells without cellular processing. Therefore, nucleocapsid and N-protein share superantigen characteristics with TSST-1. The superantigen-like

TABLE 1 T-cell proliferation in response to rabies nucleocapsid, N-protein, TSST-1 and tetanic toxoid

	Nucleo- capsid	N-protein	TSST-1	TT
T	241 ± 26	237 ± 23	238 ± 29	187 ± 26
B	352 ± 22	382 ± 34	316 ± 32	285 ± 26
T+B	3,276 ± 128	3,423 ± 136	3,874 ± 172	3,793 ± 134
T+B glutaraldehyde	3,314 ± 245	3,212 ± 97	3,645 ± 121	314 ± 68
T+B paraformaldehyde	3,615 ± 182	3,617 ± 216	3,272 ± 218	298 ± 32

B and T cells were separated by Percoll from heparinized PBL of rabies-unprimed individuals. B cells were either untreated or fixed in 1% paraformaldehyde or glutaraldehyde-phosphate buffer saline for 5 min at room temperature, washed three times and incubated with antigen (nucleocapsid, N-protein, TSST-1 or TT at $5 \mu\text{g ml}^{-1}$) for an hour at 37°C before addition of 2×10^5 purified T cells (B:T ratio of 1:10). Proliferation was measured by ^3H -thymidine incorporation (c.p.m.) after 9 days of culture. Recombinant interleukin-2 was added on day 4.

properties of the rabies nucleocapsid seem to be concentrated on the N-protein. No T-cell response was observed with mock-infected cells (hamster cells for nucleocapsid and insect cells for recombinant N-protein) treated with the same purification procedure, indicating that potential cellular contaminants are not responsible for the superantigen-like activity (data not shown).

To study the specificity of nucleocapsid and N-protein for human T-cell receptors (TCR) with particular V β s, cells from unprimed and rabies-primed individuals were analysed for V β expression after various stimulations (Table 2). PBL of unprimed donors were stimulated with N-protein (N), nucleocapsid (NC) or were not stimulated (–N), stained with anti-V β monoclonal antibodies and analysed by flow cytometry. Data are shown for six representative donors (Table 2a). For each individual, N-protein and nucleocapsid significantly increased the percentage of T cells bearing V β 8, whereas T cells bearing other V β s were unchanged. PBL of primed individuals were stimulated with rabies virus (V), phytohaemagglutinin (PHA) or nucleocapsid, then stained and analysed for V β usage. Data are shown for

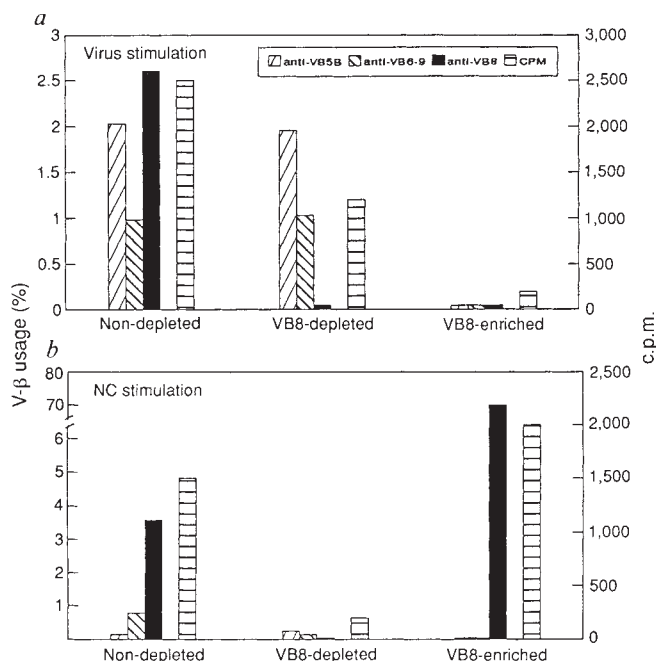


FIG. 1 Depletion of V β 8 T lymphocytes abolishes nucleocapsid-specific proliferation. PBL were incubated with anti-V β 8 antibody, washed and re-incubated with Dynabeads coated with sheep-anti-mouse IgG antibodies. Cells attached to the beads were isolated with a magnet. Non-depleted PBL, VB8-depleted PBL and VB8-positive T cells were maintained in culture with virus (a) or nucleocapsid (b) without addition of APCs. Recombinant interleukin-2 was added on day 4. Staining with anti-V β 5b, 6–9 and 8 monoclonal antibodies was done on day 10. Proliferation assays, done as previously described¹⁹, were measured on day 8.

six representative donors (Table 2b). Identical V β patterns were observed when cells were stimulated with virus or PHA, indicating that the virus does not engage T cells that carry particular TCR V β s. In contrast, as already observed with unprimed donors, the percentage of V β 8T cells was increased after nucleocapsid stimulation. Data from Tables 1 and 2 show that

TABLE 2 Selective expansion of V β 8 T-cells after *in vitro* nucleocapsid or N-protein stimulation

(a) Unprimed donors																	
	DON 1		DON 2			DON 3			DON 4			DON 5			DON 6		
	−N	+N	−N	+N	+NC	−N	+N	+NC	−N	+N	+NC	−N	+N	+NC	−N	+N	+NC
Vβ 5a	3.2	4.3	2.4	2.2	2.2	3.1	3.3	3.1	3.2	2.5	2.7	2.3	2.6	3.0	2.3	2.4	2.9
Vβ 5b	—	—	—	—	—	—	—	—	—	—	—	1.1	0.8	1.3	1.2	0.8	0.9
Vβ 6–7	4.1	4.2	2.6	2.1	2.3	1.7	1.7	1.8	1.9	1.6	1.4	4.5	3.6	3.6	0.4	0.6	0.7
Vβ 8	3.6	8.4	2.2	5.6	5.5	2.4	6.2	5.8	3.1	6.5	6.1	4.5	9.1	9.0	1.8	3.9	4.3
Vβ 12	5.7	4.9	2.2	2.3	2.3	1.3	1.5	1.2	2.8	3.5	0.9	5.2	4.2	4.6	1.6	1.5	1.5
(g) Rabies-primed donors																	
	DON 1			DON 2			DON 3			DON 4			DON 5			DON 6	
	V	PHA	NC	V	PHA	NC	V	PHA	NC	V	PHA	NC	V	PHA	NC	−N	+N
Vβ 5a	2.4	2.2	2.0	0.8	0.7	0.6	2.2	3.0	3.4	2.6	2.4	1.1	2.4	2.6	1.6	4.7	4.3
Vβ 5b	0.7	0.9	1.0	—	—	—	1.9	2.0	2.4	1.9	2.0	1.4	—	—	—	—	—
Vβ 6–7	2.3	2.3	1.9	2.0	2.8	1.6	2.3	2.7	2.7	3.2	2.5	1.6	2.0	2.3	2.0	2.3	3.1
Vβ 6–9	—	—	—	—	—	—	3.5	2.9	1.2	2.9	3.2	2.2	1.0	0.2	0.2	—	—
Vβ 8	3.4	4.0	9.4	3.4	3.2	7.0	5.0	5.6	10.2	4.8	5.3	11.4	3.2	2.8	4.9	4.5	8.2
Vβ 12	2.4	2.7	1.1	4.0	3.9	2.4	0.8	1.4	1.0	2.8	3.5	0.9	1.2	1.0	0.7	5.1	4.8

V β usage in PBL from six unprimed donors (a) and six primed (rabies vaccinated) donors (b) was estimated by cytofluorimetry before stimulation (–N) or after stimulation (day 9) with $5 \mu\text{g ml}^{-1}$ recombinant N-protein, nucleocapsid (NC), rabies virus (V) or with PHA. Recombinant interleukin-2 (5 IU ml^{-1}) was added on day 4 and 8. Cells (2×10^6) were incubated at 4°C with anti-V β antibodies or anti-TCR antibody and FITC-(Fab')₂-anti-mouse IgG and analysed with an EPICS-752 flowcytometer gated to exclude non-viable cells. Viable cells (1×10^4) were accumulated for histograms using logarithmic amplification of fluorescence intensity. Background was set at 0.20%. Usage of a given V β was expressed as a percentage of TCR-positive T cells determined with the anti-TCR antibody, WT31. Anti-V β antibodies were from T-cell Sciences (anti-V β 5a, 1C1; anti-V β 5b, W112; anti-V β 6, OT145; anti-V β 8, 16G8; and anti-V β 12, S511). The antibody V β 6–9 was a gift from O. Acuto. Irrelevant IgGs were used as negative controls. Separation of PBL, nucleocapsid and N-protein preparations were done as previously described^{10,11,19}.

nucleocapsid and N-protein do not require processing to stimulate T cell members of the V β 8 family and seem to act as superantigens.

The V β 8-positive T cells were removed from the PBL by using the anti-V β 8-specific antibody coupled to magnetic beads. V β usage and proliferation were measured in V β 8-depleted PBL, enriched-V β 8 T cells and non-depleted PBL cultivated with virus or nucleocapsid (Fig. 1). Non-depleted and V β 8-depleted PBL responded to the virus with decreased proliferative response of the depleted population corresponding to the absence of V β 8 T cells (Fig. 1*a*). The nucleocapsid induced a powerful proliferation response of PBL which was abolished by the depletion of V β 8 lymphocytes (Fig. 1*b*). The lack of proliferation of the V β 8-depleted PBL indicates that V β 8-negative T-cell subpopulations do not respond to nucleocapsid under these conditions.

The V β -enriched T cells attached to the beads were cultured with virus or nucleocapsid without addition of antigen presenting cells (APCs). Proliferation was only observed after nucleocapsid stimulation, confirming that, unlike virus, processing is not required for the presentation of nucleocapsid to T cells. A double signal provided by the anti-V β 8 antibody and

the nucleocapsid could stimulate the T cells through an unconventional pathway previously suggested for microbial toxin superantigen¹². But activated human T cells can express class II molecules of the major histocompatibility complex (MHC) which are essential for superantigen binding and subsequent T-cell proliferation. The purification procedure and culture conditions may induce MHC class II expression on V β 8 T cells which could then function as APCs.

N-protein and nucleocapsid-binding to class II $\alpha\beta$ complexes of human cell lines were analysed by flow cytometry (Fig. 2*A*). Nucleocapsid (left panels) and N-protein (right panels) could bind to class II-positive cell lines but not to a class II-negative-mutant lacking the three class II isotypes. In addition, nucleocapsid and N-protein could bind to B-cell mutants that only express HLA-DQ or DP isotypes. Nucleocapsid and N-protein also bind to the cell surface of several B-cell lines with different HLA types (data not shown). Therefore nucleocapsid and N-protein seem to use a binding site on MHC class II molecules that shows little variation among isotypes or alleles. These data, along with the indication that nucleocapsid and N-protein do not require processing to stimulate V β 8 T cells,

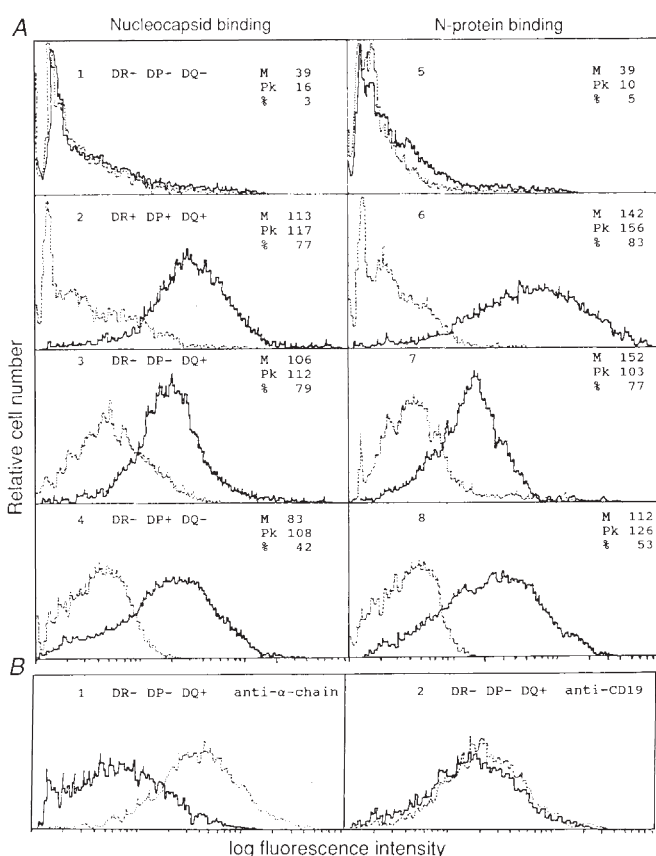
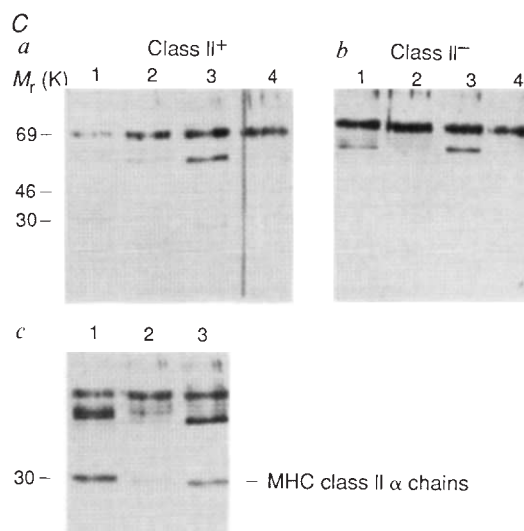


FIG. 2 *A*, MHC class II molecule expression is required for cell surface nucleocapsid-binding and N-protein-binding. Nucleocapsid- and N-protein-binding was determined by surface immunofluorescence on human MHC class II negative 721 $\times$ CEM-T2 cell line²⁰ and on class II positive B cell line 309, DP+ cell line 721-84-5 (ref. 21) and DQ+ cell line CL-13 mutants²². Cells (2×10^6) were incubated at 4 °C for 60 min, with 10 μ g nucleocapsid (left panels) or with 10 μ g of N-protein (right panels), anti-N-protein antibody (PVA3)²³ and FITC F(ab')₂ goat anti-mouse IgG. Binding was analysed in an EPICS flowcytometer as follows: results are presented as mean (M) and peak (Pk) of fluorescence for each profile obtained after incubation with NC or N-protein (solid lines). Percentages (%) of positive cells were obtained after subtraction of negative controls, without NC or N-protein incubation (dashed lines). The mean and the peak (M, Pk) in negative controls were, respectively: 38,14 (panel 1); 42,11 (panel 2); 61,64 (panel 3); 50,63 (panel



4); 33,12 (panel 5); 29,14 (panel 6); 47,13 (panel 7); and 50,63 (panel 8). The 309 cell line is DR13/7, DQ1/2. The 721-84-5 cell line is DP2/- and CL-13 DQ1/1. *B*, N-protein binding is inhibited by anti-MHC class II α -chain. Left, Binding of biotinylated N-protein on surface of DQ+ cell-line CL-13 was analysed by cytofluorimetry after cells were incubated for 30 min at 37 °C with an anti-DQ- α -chain L2 (solid line) or with medium (dashed line). Preincubation with anti-DQ- α -chain inhibited N-protein binding by 86%, whereas preincubation with anti-CD19 antibody (right) did not inhibit N-protein binding. The mean and the peak of fluorescence and the percentage of positive cells were, respectively: 131; 113; 98.3% for biotinylated N-protein binding without antibody and 82; 59; 80.1% for N-protein binding after anti-DQ- α -chain antibody incubation. *C*, Nucleocapsid binds to MHC class II α -chains. Lysates of class II-positive cells 309 (*a*) and class II-negative cells 174 $\times$ CEM-T2 (*b*) were immunoprecipitated with anti-DR complex D1-12 (ref. 24) in lane 1, monomeric anti- β -chains 2-06 (ref. 25) in lane 2, anti-DP B7-21 (ref. 26) in lane 3 and with anti-class I W6-32 (ref. 27) in lane 4. The immunoprecipitates were separated on SDS-PAGE, transferred to nitrocellulose and probed with biotinylated-nucleocapsid. After detection of nucleocapsid-binding with streptavidin-horseradish peroxidase and ABTS (blue colour), the blot from *a* was reincubated with the anti- α -chain antibody, 5-F2-3, and developed with diaminobenzidine to obtain a brown colour (*c*). Under these conditions, the 30K protein bound by nucleocapsid was identified as the HLA class II α -chains. Two proteins were nonspecifically recognized by nucleocapsid in class I and class II immunoprecipitates from normal and class II negative-mutant cell lines. These proteins are not, therefore, the nucleocapsid-specific receptors and may correspond to serum albumins and heavy chains of IgG. SDS-PAGE, electrotransfer, immunoblotting and immunoprecipitation were done as previously described^{28,29}.

strongly suggest that nucleocapsid and N-protein bind directly to MHC class II molecules to form a complex that can interact with the TCR in an MHC unrestricted fashion.

Inhibition of N-protein binding was tested with several anti-class II antibodies. A strong inhibitory activity was obtained with anti-DQ- α -chain antibodies (Fig. 2B). A weak inhibition was observed with a monomorphic anti- β -chain antibody and N-protein binding was markedly reduced by an anti-DR complex antibody, DI-12 (data not shown). This antibody mainly recognizes mature $\alpha\beta$ complexes lacking the invariant chain which are likely to contain a peptide acquired in endocytic vesicles suggesting that nucleocapsid and N-protein, like other superantigens, could interact with MHC class II molecules outside the antigen groove. The direct binding capacity of biotinylated nucleocapsid to α - and β -chains was studied by western blot on immunoprecipitated MHC class II molecules (Fig. 2C). Nucleocapsid bound a 30K protein in DR and DP precipitates from normal B cells (Fig. 2Ca) but not from the class II negative mutant (Fig. 2Cb). A subsequent incubation of the same blot with an anti-MHC class II α -chain antibody identified this protein as the DR- α and DP- α chains (Fig. 2Cc). Nucleocapsid and the anti- α -chain antibody reacted very weakly with the α -chains immunoprecipitated with a monomorphic anti- β -chain antibody (lane 2, Fig. 2Ca). The MHC molecules immunoprecipitated by this antibody are mainly associated with the invariant chain and represent immature class II complexes (data not shown). The nucleocapsid-binding capacity may depend on the post-translational modifications of the chains during the intracellular transport of the $\alpha\beta$ complexes. Altogether, these data indicate that nucleocapsid binds to cell surface MHC class II α -chains. Similar binding capacity has been suggested for the bacterial superantigen TSST-1¹³ and the *Mycoplasma arthritidis* mitogen MAM¹⁴.

Superantigen-like properties of rabies virus nucleocapsid and N-protein could have considerable effects on vaccinal strategy. Nucleocapsid and N-protein of rabies virus, like other internal antigens (influenza, measles) can protect animals and enhance the production of neutralizing antibodies directed to the viral envelope proteins^{9,15-18}. This could reflect the expansion of CD4⁺ V β 8 T lymphocytes involved in the production of virus neutralizing antibodies. But because clonal expansion of T cells could be followed by anergy, further *in vivo* studies are necessary to characterize long-term protection. □

Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum

Kirsten Sandvig*, Øystein Garred*, Kristian Prydz*, Juri V. Kozlov†, Steen H. Hansen‡ & Bo van Deurs‡

* Institute for Cancer Research at the Norwegian Radium Hospital, Montebello, 0310 Oslo 3, Norway

† W. A. Engelhardt Institute for Molecular Biology, Academy of Sciences of Russia, Vavilov str 32, 117984 Moscow B334, Russia

‡ Structural Cell Biology Unit, Department of Anatomy, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

SHIGA toxin and some other protein toxins that act on targets in the cytosol have previously been shown to enter the *trans*-Golgi network¹⁻⁹. Transport by this route may be necessary for translocation of the toxin to the cytosol and for intoxication⁵⁻⁹, but it is not known whether the enzymatically active part of the toxins actually enters the cytosol from the *trans*-Golgi network. It has been suggested that such toxins are transported in a retrograde manner to the endoplasmic reticulum and that translocation occurs in this organelle¹⁰, but retrograde transport of endocytosed material beyond the *trans*-Golgi network has never been demonstrated. Here we show that in butyric acid-treated A431 cells endocytosed Shiga toxin is not only transported to the *trans*-Golgi network, but also to all Golgi stacks, to the endoplasmic reticulum and to the nuclear envelope. Furthermore, butyric acid sensitizes the cells to Shiga toxin, which is consistent with the possibility that retrograde transport is required for translocation of the toxin to the cytosol.

A431 cells bind and internalize Shiga toxin but are completely resistant to it. Butyric acid sensitizes MCDK cells to Shiga toxin⁴, so we tested whether A431 cells could also be sensitized by this compound and found that incubation of A431 cells with butyric acid for 48 h did indeed sensitize the cells to the toxin (Fig. 1). Cells not exposed to butyric acid were not affected by 1,000 ng ml⁻¹ Shiga toxin, but protein synthesis in butyric acid-treated cells was reduced by half with as little as 2 ng ml⁻¹ of toxin. Short incubation times with butyric acid had no sensitizing effect (data not shown).

Incubation of A431 cells with ¹²⁵I-labelled Shiga toxin at 37 °C showed that butyric acid treatment increased by 3–4 times

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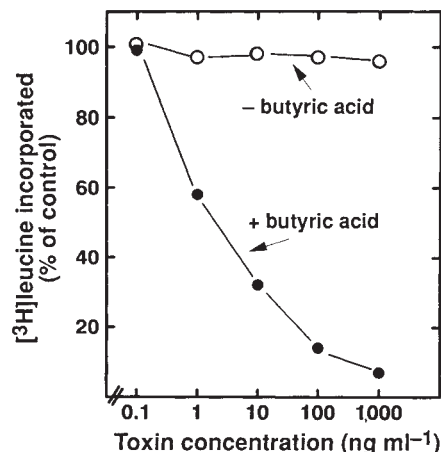


FIG. 1 Ability of butyric acid to sensitize A431 cells to Shiga toxin. A431 cells growing in 24-well disposable plates were incubated for 48 h with and without 2 mM butyric acid. Increasing concentrations of Shiga toxin were added and protein synthesis measured 4 h later as described⁴.

the amount of cell-associated toxin (data not shown). Furthermore, subcellular fractionation (data not shown) indicated that in untreated cells very little or no Shiga toxin is transported to the Golgi apparatus, whereas the percentage of cell-associated Shiga toxin present in the Golgi fractions was higher in butyric acid-treated cells than in untreated cells. Altogether, butyric acid seems to increase the amount of Shiga toxin transported to the Golgi compartment about 10 fold.

To visualize Shiga toxin ultrastructurally we used a Shiga toxin-horseradish peroxidase conjugate³ which bound specifically to the surface of both untreated and butyric acid-treated A431 cells. In the untreated cells the conjugate could be traced to endosomes/lysosomes, whereas very little, if any, conjugate was delivered to Golgi cisterns and Golgi-associated cisternal structures that we consider to be the *trans*-Golgi network^{1,3,4,7} (Fig. 2a and b). After a 48-h incubation with butyric acid, binding of the Shiga toxin-horseradish peroxidase conjugate to the cell surface was more pronounced, and also

the labelling of endosomes/lysosomes was dramatically increased (Fig. 2c). Most importantly, several Golgi complexes now showed a marked labelling of all stacks (Figs. 2c and 3) and endoplasmic reticulum (ER) cisterns, and in some cases even nuclear envelopes were distinctly labelled by the Shiga toxin conjugate (Fig. 3). Labelling was somewhat heterogeneous as in the same experiment we also observed cells with little or no labelling of the Golgi complex and with apparently unlabelled ER cisterns. Possibly, the toxin-horseradish peroxidase conjugate, in amounts below the level of cytochemical detection, had reached the Golgi stacks and the ER in these cells. Thus, protein synthesis in the whole population of butyric acid-treated cells was inhibited by Shiga toxin (Fig. 1).

To our knowledge this is the first demonstration of transport of an endocytosed ligand to the Golgi cisternae, the ER and to the nuclear envelope. It has been reported that endocytosed simian virus 40 (SV40) is transported to the ER in CV-1 cells¹¹, but the virus seems to follow a different route: unlike Shiga toxin, which enters from clathrin-coated pits at the cell surface^{3,4}, SV40 is internalized in small uncoated vesicles and is delivered directly to the ER and so is not observed in Golgi stacks. It has been proposed that the KDEL receptor may bring proteins back to the ER not only from an intermediate compartment between ER and Golgi, but possibly also from the *cis*-Golgi and other Golgi stacks^{12,13}. This receptor could play a part in the intoxication of cells with pseudomonas exotoxin A and other protein toxins that have a carboxy-terminal sequence recognized by the KDEL receptor^{10,14,15}. We have shown here that a protein toxin can be transported all the way from the cell surface to the ER and that this transport occurs concomitantly with a sensitization of the cells to the protein toxin. This occurs in spite of the lack of a KDEL sequence in Shiga toxin¹⁶⁻¹⁸, although the toxin might interact with proteins containing a KDEL-like sequence.

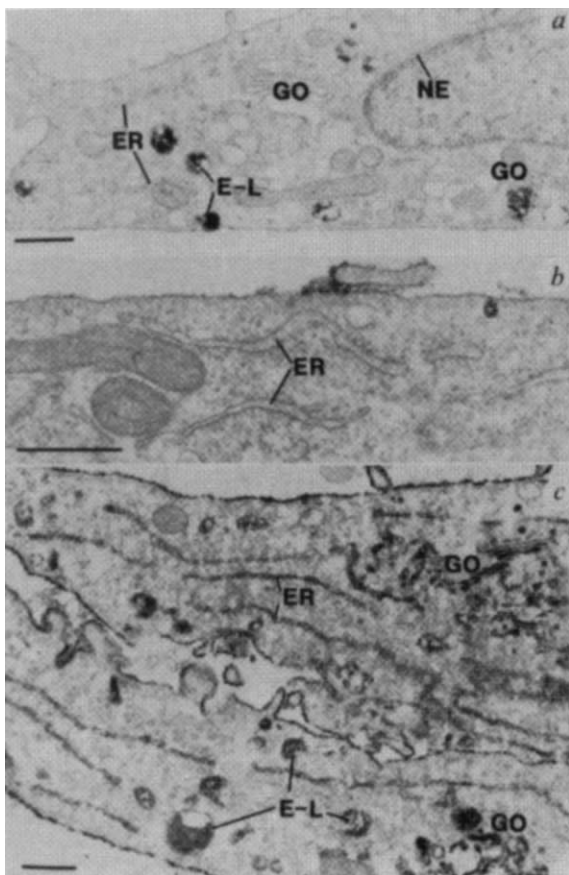


FIG. 2 Localization of endocytosed Shiga toxin-horseradish peroxidase (HRP) in A431 cells. *a*, Low magnification electron micrograph of an A431 cell incubated with Shiga-HRP for 60 min at 37 °C. The toxin conjugate is seen in endosomes/lysosomes, while the Golgi complexes, the ER and the nuclear envelope appear unlabelled. *b*, Higher magnification of a cell incubated with Shiga-HRP for 60 min at 37 °C, showing unlabelled endoplasmic reticulum cisterns. *c*, Low-power view of A431 cells incubated with butyric acid before exposure to Shiga-HRP. In addition to labelling of endosomes/lysosomes, Golgi complexes and ER cisterns are also distinctly labelled. Scale bars, 0.5 µm. ER, endoplasmic reticulum; E-L, endosome/lysosome; GO, Golgi complex; NE, nuclear envelope.

METHODS. A431 cells were either incubated directly with Shiga-HRP for 60 min at 37 °C (*a*, *b*), or first incubated with 2 mM butyric acid for 48 h at 37 °C and then with Shiga-HRP. The Shiga-HRP conjugate was prepared as described³. After incubation with toxin conjugate, cells were washed, fixed with glutaraldehyde, treated with diaminobenzidine-H₂O₂ and processed for electron microscopy^{3,7}.

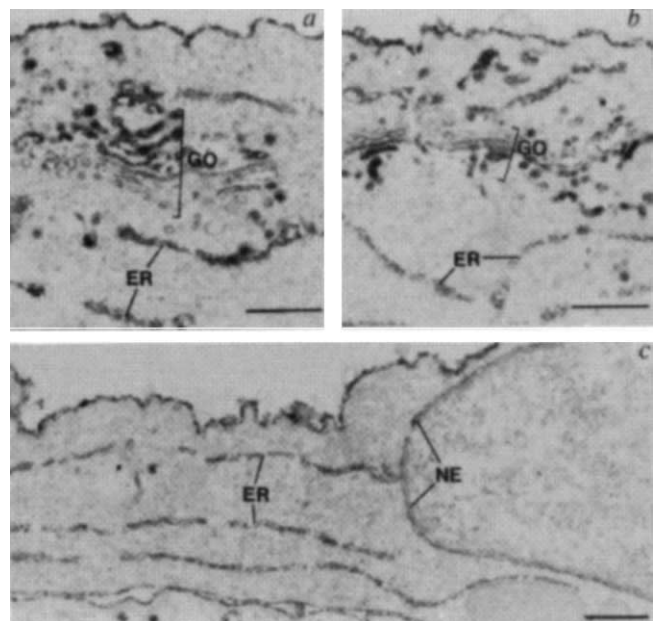


FIG. 3 Localization of endocytosed Shiga toxin-HRP to the Golgi complex, the endoplasmic reticulum and the nuclear envelope of butyric acid-treated A431 cells. *a* and *b*, Toxin conjugate is present in all stacks of the Golgi complex as well as in ER cisterns. *c*, Example of Shiga-HRP present in parallel cisterns of ER as well as in the nuclear envelope. Scale bars, 0.5 µm. ER, endoplasmic reticulum; GO, Golgi complex; NE, nuclear envelope.

METHODS. A431 cells were incubated with 2 mM butyric acid for 48 h at 37 °C. Then Shiga-HRP prepared as described³ was added at 37 °C. After 60 min cells were washed, fixed, treated with diaminobenzidine-H₂O₂ and processed for electron microscopy^{3,7}.

It is not clear whether the butyric acid-induced overall increase in toxin uptake and transport to the *trans*-Golgi network allows us to visualize a retrograde transport that also occurs in untreated A431 cells, or whether butyric acid treatment somehow induces a retrograde flow that is blocked in untreated cells. It may be that retrograde transport of molecules from the cell surface to the ER occurs to a limited extent even in untreated cells. □

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A role for ADP-ribosylation factor in nuclear vesicle dynamics

Annette L. Boman, Timothy C. Taylor*,
Paul Melançon* & Katherine L. Wilson†

Department of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205, USA

* Department of Chemistry and Biochemistry, University of Colorado at Boulder, Boulder, Colorado 80309, USA

Two distinct steps in nuclear envelope assembly can be assayed *in vitro*^{1–3}: the protein-mediated binding⁴ of nuclear-specific vesicles to chromatin, and the subsequent fusion⁵ of these vesicles to enclose the chromatin within a double nuclear membrane. Nuclear vesicle fusion, like fusion in the secretory pathway^{6,7}, requires ATP^{8,9} and cytosol^{1,3,5} and is inhibited by nonhydrolysable GTP analogues^{1,2}. The sensitivity of nuclear vesicle fusion to GTP- γ S requires a GTP-dependent soluble factor, the properties of which are strikingly similar to a GTP-dependent Golgi binding factor (GGBF) that inhibits Golgi vesicle fusion in the presence of GTP- γ S and belongs to the ADP-ribosylation factor (ARF) family of small GTPases^{10,11}. In the presence of GTP- γ S, ARF proteins and α -, β -, γ -, δ -COP ('coatomer') subunits are associated with Golgi transport vesicles^{6,12,13}, but the exact roles of ARF proteins in secretion are not yet understood. We report here that purified ARF1 and GGBF have GTP-dependent soluble factor activity in the nuclear vesicle fusion assay. Our results show that the function of ARF is not limited to the Golgi apparatus, and indicate that there may be a link between the formation of nuclear vesicles during mitosis and proteins involved in secretion.

To determine whether GTP-dependent soluble factor (GSF) and ADP-ribosylation factor are related, we tested ARF proteins

from two sources, recombinant ARF1 (ref. 14) and purified GTP-dependent Golgi binding factor¹⁰, for GSF activity. *Xenopus* membranes were incubated with chromatin to allow the binding of nuclear-specific vesicles; fusion was then stimulated by the addition of cytosol and monitored by light microscopy¹. To assay GSF (inhibition of fusion) activity, membranes were preincubated with the putative GSF and GTP- γ S, and then washed before being tested for binding and fusion.

Amino-terminal myristoylation is required for ARF1 to bind membranes in the presence of GTP- γ S (ref. 15). We therefore tested both myristoylated (mARF) and non-myristoylated (non-mARF) bacterially expressed recombinant mammalian ARF1 (provided by R. Kahn) for GSF activity (Fig. 1). Pretreatment of membranes with mARF plus GTP- γ S inhibited nuclear vesicle fusion, indicated by smaller nuclear envelope surface area. In contrast, mARF plus GTP did not have GSF activity, nor did non-mARF plus either GTP- γ S or GTP. These controls showed that ARF's ability to provide GSF activity required GTP- γ S and N-terminal myristoylation of the ARF1 protein. None of these treatments interfered with vesicle binding to chromatin (ref. 1, and data not shown).

As a further test for the GSF activity of ARF proteins, we tested two physically distinct purified proteins, GGBF and GGBF*, from bovine brain cytosol¹⁰. GGBF and GGBF* have the same M_r of 20,000 (20K) and have been identified as ARFs by immunological and biochemical criteria. Both are recognized

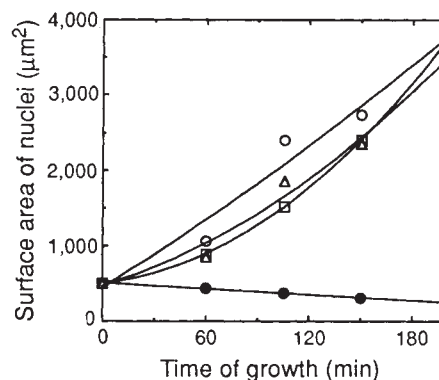


FIG. 1 Myristoylated ARF1 has GSF activity. Membranes were pretreated with GTP- γ S or GTP and bacterially expressed recombinant mammalian ARF1, either myristoylated (mARF) or non-myristoylated (non-mARF). Only mARF plus GTP- γ S (●) inhibited fusion of nuclear vesicles; membranes pretreated with non-mARF plus GTP (□) or GTP- γ S (○), or with mARF plus GTP (△), fused normally.

METHODS. Membranes and cytosol were prepared from *Xenopus* egg extracts as described¹. Myristoylated ARF is obtained as a mixture of mARF (40%) and non-mARF (60%) from bacterial cells cotransformed with the ARF1 cDNA and the myristate transferase gene¹⁴. To assay nuclear vesicle fusion, membranes were pretreated as follows: 30- μ l reactions containing 3 μ l membranes (~30 mg ml⁻¹), 7 μ g ARF1, 1 mM GTP- γ S or GTP (Boehringer Mannheim), and membrane-wash buffer (250 mM sucrose, 2.5 mM MgCl₂, 50 mM KCl, 50 mM HEPES, pH 8.0, 1 mM dithiothreitol, 0.5 mM ATP, 1 μ g ml⁻¹ each of aprotinin and leupeptin) were incubated for 30 min at room temperature. Each reaction was diluted with an equal volume of membrane-wash buffer, then centrifuged at 25,000g for 15 min to pellet the membranes. Resuspended membranes were added to demembrated sperm chromatin³⁰ to allow binding of nuclear-specific vesicles¹. Fresh cytosol was added to the binding reactions after 30 min to stimulate vesicle fusion and nuclear envelope growth. Aliquots were monitored for fusion by light microscopy using phase contrast and fluorescence of the lipid dye dihexyloxacarbocyanine¹. The extent of fusion was quantitated by assuming that fusion is proportional to nuclear envelope growth. The diameters of at least 10 nuclei per timepoint were measured using an ocular micrometer; the average nuclear envelope surface areas and standard deviations (s.e.) were calculated and graphed as a function of time after the addition of cytosol. This experiment was typical of several repeats, and in this case the s.e. for the last time points were: ●, 70 μ m²; △, 800 μ m²; ○, 700 μ m²; □, 300 μ m².

† To whom correspondence should be addressed.

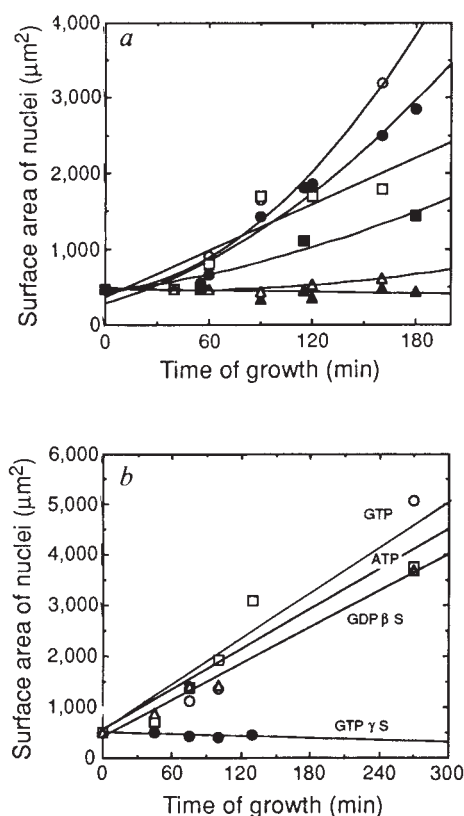


FIG. 2 Purified GGBF has GSF activity. *a*, GGBF and GGBF* are titratable during pretreatment. 0.17 μg GGBF (●) had little effect on fusion relative to the controls lacking GGBF (○), whereas increasing amounts of GGBF delayed (0.51 μg ; ■) or completely inhibited (1.7 μg ; ▲) fusion and nuclear envelope growth. Greater concentrations of GGBF* were required for delayed (1.7 μg ; ◊) or complete (5.1 μg ; △) inhibition of fusion. Standard errors for the last time points: circles, 700 μm^2 ; squares, 450 μm^2 ; triangles, 100 μm^2 . *b*, Inhibition of nuclear vesicle fusion by GGBF is GTP- γ S-specific. *Xenopus* membranes pretreated with purified GGBF and 1 mM GTP- γ S (●) were unable to fuse, whereas membranes pretreated with GGBF and 1 mM GTP (○), GDP- β S (△), or ATP (◊) fused and grew normally. Standard error for the last time points: GTP- γ S, 65 μm^2 ; others, 700–900 μm^2 .

METHODS. As for Fig. 1, using 1 mM GTP- γ S and 0.17 to 5.1 μg purified bovine brain GGBF or GGBF* during pretreatment.

by antibodies that crossreact with ARF1 and ARF3, but not by antibodies against ARF4, and both are active when assayed for ADP-ribosylation cofactor activity¹⁶. The GGBF fraction contains about 25% GGBF* protein but is 3 times as active as pure GGBF* in the Golgi fusion assay. *Xenopus* nuclear vesicles preincubated with GTP- γ S and purified GGBF failed to fuse, showing that purified GGBF has GSF activity (Fig. 2). Lower concentrations of GGBF (Fig. 2*a*) and mARF1 (data not shown)

resulted in less inhibition of fusion just as *Xenopus* GSF activity depended on the amount of cytosol used to pretreat membranes¹. Furthermore, GGBF was 2- to 3-fold more active than GGBF* because higher concentrations of GGBF* (5.1 μg per reaction versus 1.7 μg GGBF) were required to inhibit nuclear vesicle fusion fully (Fig. 2*a*). This paralleled their relative activities in the Golgi fusion assay. The observed GSF activity of GGBF was dependent on GTP- γ S, because membranes preincubated

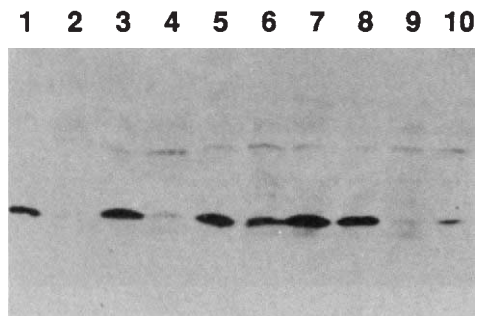


FIG. 3 Western blot probed with monoclonal antibody 1D9 against mammalian ARF1 shows that a soluble 20K *Xenopus* protein becomes associated with membranes during pretreatment with 1 mM GTP- γ S. Lane 1, *Xenopus* cytosol (100 μg); lane 2, *Xenopus* membranes (90 μg); lane 3, 90 μg *Xenopus* membranes pretreated with *Xenopus* cytosol and GTP- γ S; lane 4, 90 μg *Xenopus* membranes pretreated with *Xenopus* cytosol and GTP; lane 5, 90 μg *Xenopus* membranes pretreated with GGBF and GTP- γ S; lane 6, 90 μg *Xenopus* membranes pretreated with cytosol and 10 μg ml⁻¹ brefeldin A (in methanol; Sigma) for 10 min. GTP- γ S was then added and incubated for 20 min; lane 7, 90 μg *Xenopus* membranes pretreated with cytosol and GTP- γ S for 20 min, then 10 μg ml⁻¹ brefeldin A was added for 10 min; lane 8, nuclear-specific membranes pretreated with *Xenopus* cytosol and GTP- γ S; lane 9, nuclear-specific membranes pretreated with *Xenopus* cytosol and GTP; lane 10, nuclear-specific membranes pretreated with brefeldin A as in lane 6.

METHODS. Nuclear-specific membranes were obtained by allowing 30 μl membranes to bind to 25 μl (40,000 per μl) sperm chromatin. The chromatin and bound membranes were pelleted through 1.3 M sucrose at 13,000*g* for 5 min. The resuspended pellet was digested with DNase (Worthington) for 10 min at room temperature. Membranes (lanes 3–10) were pretreated by incubating 4 μl membranes with 1 mM GTP- γ S or 1 mM GTP and either 30 μl cytosol or 1.7 μg GGBF for 30 min at room temperature, diluting the sample with an equal volume of membrane-wash buffer (see Fig. 1 legend), and pelleting the membranes at 35,000*g* for 15 min. All samples were loaded onto a 7–15% gradient SDS-PAGE gel, transferred to Immobilon membrane (90V, 45 min), and probed with monoclonal antibody 1D9 (1:6,700 dilution; from R. Kahn). Bound 1D9 antibodies were visualized and quantitated using horseradish peroxidase-conjugated secondary antibodies (BioRad) and enhanced chemiluminescence (ECL, Amersham).

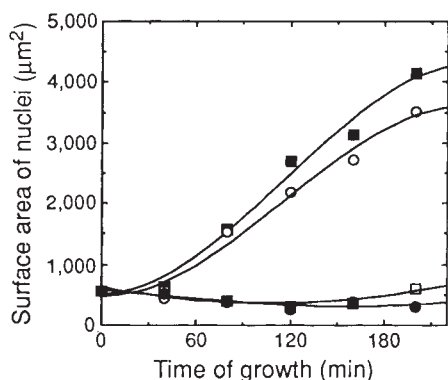


FIG. 4 Brefeldin A (BFA) blocks GSF inhibition of fusion. Concentrations of BFA $>2.5 \mu\text{g ml}^{-1}$ reduce or inhibit GSF activity; complete blocking was observed using $10 \mu\text{g ml}^{-1}$ BFA. Because GSF activity is assayed as a lack of fusion, blocking of GSF activity by BFA is indicated by increased nuclear envelope growth and larger nuclear surface area. Methanol alone had no effect on GSF activity. The s.e. for the last time points were: methanol alone (●), $100 \mu\text{m}^2$; $2.5 \mu\text{g ml}^{-1}$ BFA (□), $450 \mu\text{m}^2$; $10 \mu\text{g ml}^{-1}$ BFA (○), $700 \mu\text{m}^2$; no GTP- γ S control (■), $900 \mu\text{m}^2$.

METHODS. Membranes were preincubated with *Xenopus* cytosol and exposed to different concentrations of BFA for 10 min before addition of 1 mM GTP- γ S. Samples were processed as for Fig. 1.

with GGBF and either GTP, GDP- β S or ATP fused and grew normally (Fig. 2b). We concluded that GGBF and GGBF* may perform similar functions in the Golgi and nuclear fusion systems.

Xenopus cytosol contains a 20K protein that is recognized by a monoclonal antibody to mammalian ARF1 (Fig. 3, lane 1). This putative *Xenopus* ARF was scarce in the membrane fraction (lane 2), and became membrane-associated during a 30-min preincubation of total membranes (lanes 3 and 4) or nuclear-specific membranes (lanes 8 and 9) with cytosol and GTP- γ S (lanes 3 and 8), but not with cytosol and GTP (lanes 4 and 9). Purified GGBF also became membrane-associated after incubation with GTP- γ S (lane 5). These results supported our idea that inhibition of fusion correlates with irreversible binding of ARF-GTP- γ S to nuclear vesicles.

The fungal metabolite brefeldin A blocks the binding of ARF and β -COP to Golgi membranes and thereby prevents coat assembly and vesicular transport^{17,18}. The target of brefeldin A binding on membranes is not known¹⁹. Brefeldin A added to *Xenopus* membranes and cytosol 10 min before the addition of GTP- γ S during membrane pretreatment inhibits GSF activity (Fig. 4). This treatment also reduces the binding of endogenous ARF to total membranes by 54% (Fig. 3, lane 6) and to nuclear-specific vesicles by 75% (Fig. 3, lane 10). The extent of brefeldin A rescue depends on its concentration. When added to membranes 20 min after preincubation with cytosol and GTP- γ S, brefeldin A has no effect on GSF activity (not shown) or ARF binding (Fig. 3, lane 7). These results suggest that brefeldin prevents the irreversible binding of ARF-GTP- γ S to nuclear vesicles and thus allows vesicle fusion to proceed normally in the presence of fresh cytosol.

Synthetic peptides corresponding to amino acids 2–17 of ARF1 block ARF1 function in Golgi²⁰, endoplasmic reticulum²¹ and endosome²² fusion assays, and also block ARF cofactor activity in the ADP-ribosylation reaction (R. Kahn, personal communication). This N-terminal peptide competes with full-length ARF1 for binding to Golgi membranes²³. In contrast,

$100 \mu\text{M}$ ARF1 peptide has no effect on nuclear vesicle fusion (data not shown), perhaps as a result of species-specific or cell-type specific differences between *Xenopus* and mammalian ARFs (or ARF-binding proteins).

Our results strongly suggest that ARF proteins have GSF activity and that GSF is likely to be a member of the ARF family. Purification of *Xenopus* GSF will be necessary to identify it as a specific ARF protein(s). It is possible that ARF-GTP- γ S inhibits fusion nonspecifically as it binds spontaneously to liposomes *in vitro*¹⁵. But ARF-GTP- γ S binding to Golgi membranes is blocked by brefeldin A and seems to require specific receptors that are destroyed by trypsin treatment¹². We speculate that ARF-binding proteins may also be present on nuclear vesicles. In addition, an unidentified heterotrimeric G protein is proposed to regulate the binding of ARF and β -COP to Golgi membranes^{24,25}. G proteins can be detected by the effects of aluminium fluoride ion (AlF_n), which activates trimeric G proteins but not small GTPases¹⁵. AlF_n inhibits vesicle fusion during endoplasmic reticulum-to-Golgi²⁶ and intra-Golgi⁷ transport, and inhibits endosome fusion²⁷. Our system provides an interesting contrast¹ because the lack of sensitivity to AlF_n suggests that trimeric G proteins are either absent from nuclear vesicles, insensitive to AlF_n , or have no role in GSF binding.

We propose that GSF/ARF is the first molecular link between secretory vesicles and vesicles that mediate post-mitotic nuclear assembly. Our results are consistent with two roles for GSF: GSF as an independent negative regulator of fusion, and GSF as a component that stimulates coat assembly on vesicles. These models are not mutually exclusive. ARF-GTP- γ S binding to nuclear vesicles suggests that nuclear vesicles are sometimes coated, and is consistent with the idea²⁸ that a kinetic block to fusion during mitosis causes organelle disassembly. In this model, nuclear membrane disassembly involves the formation of interphase-like coated vesicles^{6,29}. Further experiments will be needed to test these models as there is no direct evidence that nuclear vesicles are coated during their formation in mitosis. □

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Structural basis of blue-colour development in flower petals from *Commelina communis*

Tadao Kondo*, Kumi Yoshida†, Atsushi Nakagawa‡, Takatoshi Kawai§, Hirotohi Tamura§ & Toshio Goto§||

* Chemical Instrument Center, Nagoya University, Chikusa, Nagoya 464-01, Japan

† School of Life Studies, Sugiyama Jogakuen University, Chikusa, Nagoya 464, Japan

‡ Photon Factory, National Laboratory for High Energy Physics (KEK), Tsukuba, Ibaraki 305, Japan

§ Laboratory of Organic Chemistry, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464-01, Japan

|| Deceased

FLOWER colours, from red through purple to blue, are mostly from anthocyanins, a type of flavonoid^{1–5}. Although there are many colours, only a few anthocyanidins, chromophores of the pigments, have been found. The colour of the pigments is stable in plants for a few days to one month but the extracted anthocyanins are, nevertheless, unstable and quickly lose colour by hydration in a neutral aqueous solution^{1–5}. In 1915 Willstätter proposed that flower-colours vary because anthocyanins change their colour with the pH⁶. Shibata and Shibata questioned the theory because most plant cell sap was weakly acidic or neutral. Their alternative, based on metal-complex theory⁷ was refuted by Everest⁸. In 1958 Hayashi isolated in crystal form a blue pigment⁹, commelinin, a metal-complex anthocyanin (named metalloanthocyanin)^{10,11}, from the blue petals of *Commelina communis*. But the existence of a blue-coloured magnesium complex was denied by Bayer *et al.*¹². We obtained the same blue pigment as intact commelinin by reconstruction from its components¹³. We also prepared Cd-commelinin in which the complexation metal Mg²⁺ was replaced with Cd²⁺. We report here the X-ray crystal structure of a real anthocyanin and a sugar-containing flavonoid, using Cd-commelinin. The blue flower-colour development and the stability of the colour can be explained by metal complexation of anthocyanin and intermolecular hydrophobic association.

Commelin is readily soluble in water. It cannot be dialysed⁹, and the blue colour is stable in concentrated solutions. When diluted the solution quickly becomes colourless due to decomposition, suggesting that commelinin is an associated supra molecule held together by weak hydrophobic interactions. Contrary to previous studies¹¹, we found that the anthocyanin composition of commelinin was malonylawobanin (malonylawobanin, M) and not awobanin¹⁴. We resynthesized commelinin from the isolated pure components: M, flavocommelin (F) and magnesium. The composition of the pigment was established to be [M₆F₆Mg₂]⁶⁻, indicating that a metal ion is an essential component of commelinin^{13,15}. Mg²⁺ can be replaced with other divalent metal ions¹⁶ such as Mn²⁺, Cd²⁺, Zn²⁺, Co²⁺ and Ni²⁺. In addition, many further commelinin-like complexes were obtained through replacement of M by other isolated anthocyanins^{1,4}. These replacement studies indicated that the position of the metal chelation is the *ortho*-dihydroxyl groups on the B-ring of the anthocyanin (Table 1).

The arrangement of each of the components has been studied by circular dichroism (CD, Fig. 1c) and proton nuclear magnetic resonance (¹H NMR, Fig. 2a). The CD spectra of commelinin showed a strong negative exciton-type Cotton curve, indicating a left-handed screw stacking of the chromophore of M^{17,18}. A stacking arrangement of M and F in the molecule was studied by nuclear Overhauser effect (NOE) of the ¹H NMR experiments. Strong negative NOEs (Fig. 2b) among the inter-components were observed between aromatic protons, suggesting that M and F are stacking face-to-face in a cross-parallel

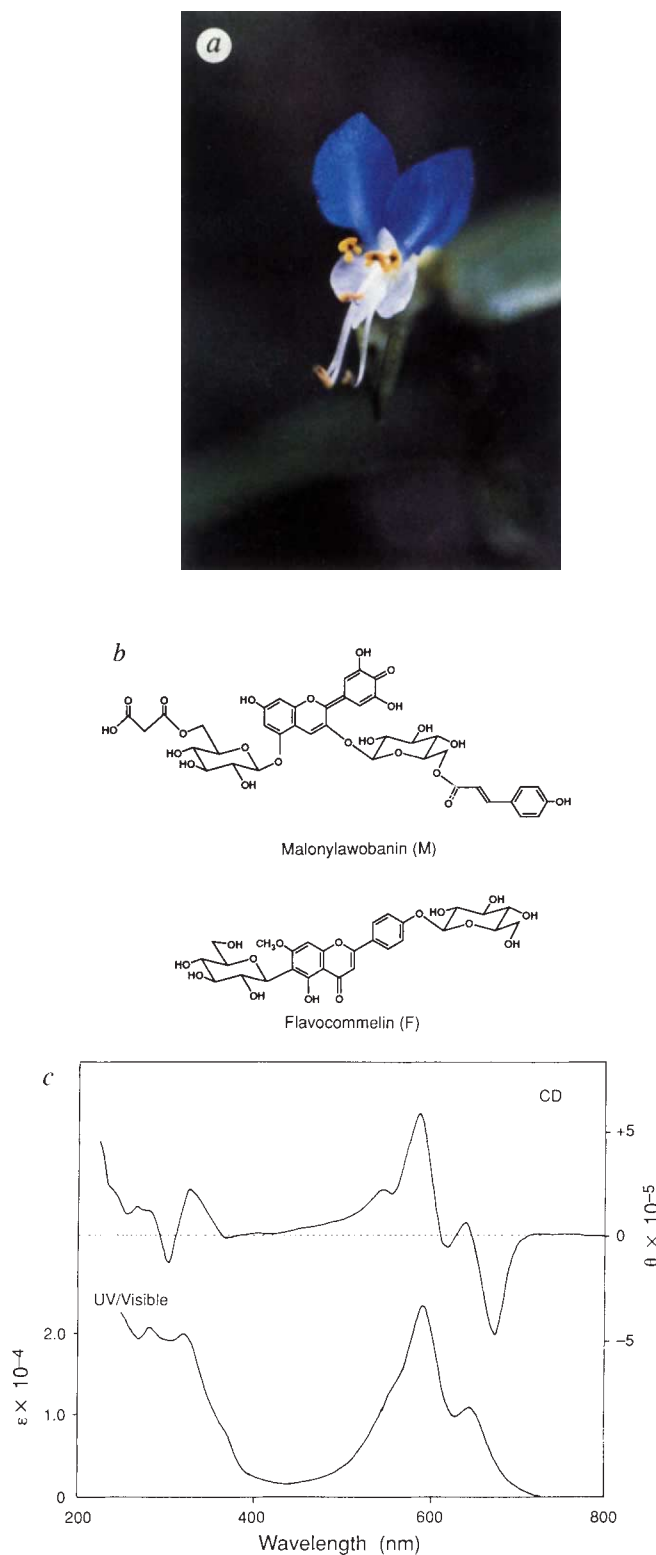
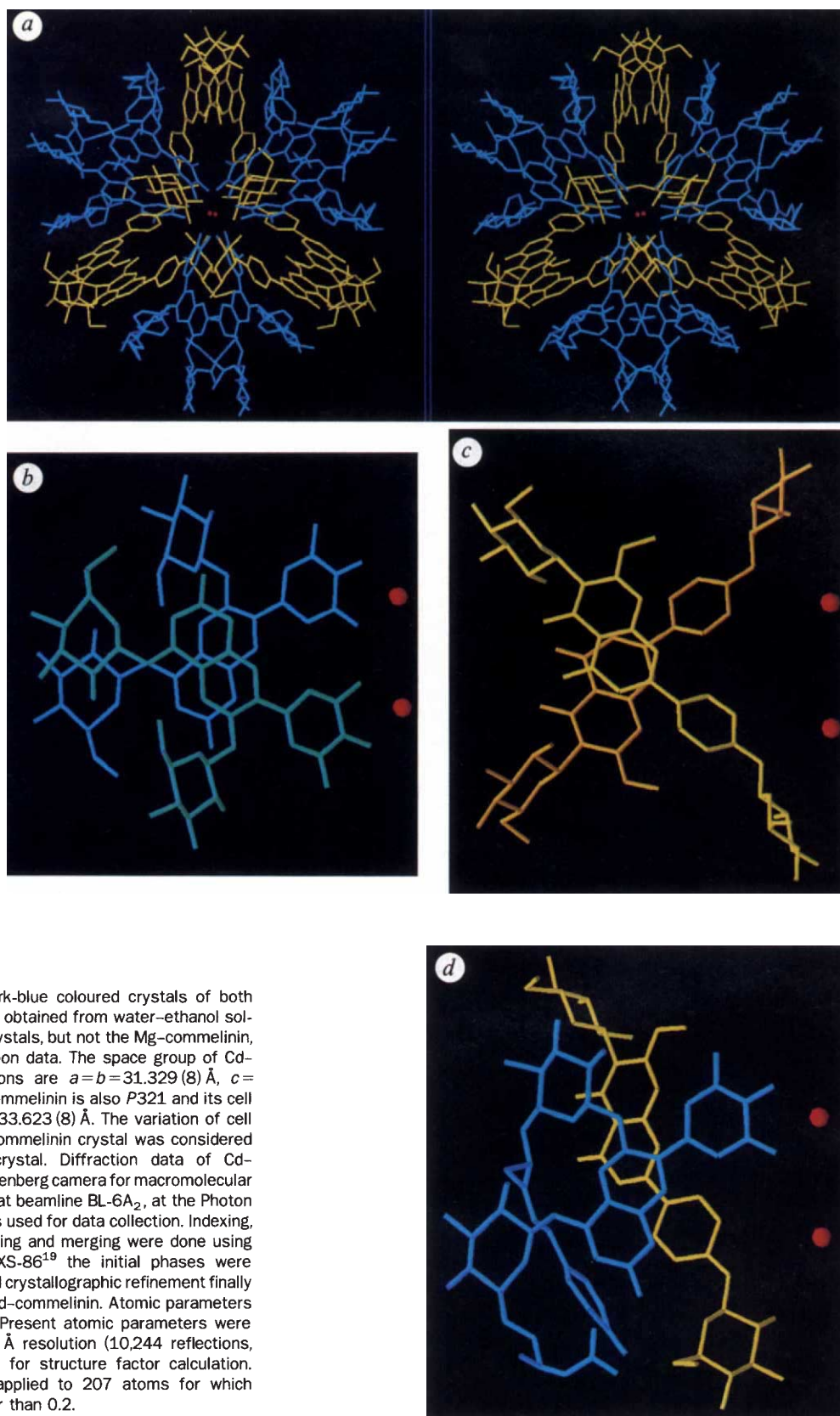


FIG. 1 a, A blue flower of *Commelina communis*. b, The anthocyanin component of commelinin is malonylawobanin (M) and the flavone component is flavocommelin (F). c, Electronic spectrum and CD of commelinin were measured at a concentration of 5×10^{-5} M in aqueous solution at pH 5.0 (cell length 1.0 mm). The λ_{vismax} of commelinin was 590 nm (ϵ ; 22,800) and 645 nm (ϵ ; 10,800). Commelinin showed blue colour, at pH 4 to 8, although anthocyanins change their colour continuously depending on the pH of the media^{1,5}. At the concentration of 5×10^{-5} M, the blue colour of commelinin was stable but at 10^{-6} M it lost colour quickly. The exciton-type Cotton effect was observed at 580 nm (θ ; +615,000) and 668 nm (θ ; -480,000). This strong negative Cotton indicates the left-handed screw stacking of the anthocyanidin nucleus^{17,18} (M and M), and that F and F is also a left-handed screw conformation²⁰.

FIG. 3 Atomic model of Cd-commelinin structure. *a*, Stereoscopic drawing of a wire model of Cd-commelinin viewed along a 3-fold axis. Water molecules are not shown. Blue, malonylawobanin (M); yellow, flavoccommelin (F); red, cadmium ions. Two cadmium ions are located at the centre of a molecule, 5.1 Å apart, and both are on a crystallographic 3-fold axis. Six Ms and six Fs surround these two cadmium ions with coordination to the M's oxygen atoms. Each of two M and F fragments in an asymmetric unit are correlated by 3-fold crystallographic symmetry. Both M and F fragments in an asymmetric unit are correlated by noncrystallographic pseudo-2-fold axis to each other. *b*, Stacking of M and M in commelinin. The acyl moieties are not shown. Blue-green, the front side molecule; dark blue, the back side molecule; red, cadmium ion. The chromophores are arranged in a left-handed screw manner and the distance of the aromatic rings of the two anthocyanin nuclei are ~ 3.5 Å. *c*, Stacking of F and F in commelinin. Yellow, the front side molecule; orange, the back side molecule; red, cadmium ions. The chromophores are arranged in a left-handed screw manner, and the distance between the aromatic rings of the two flavonoids is ~ 3.5 Å. The planes of the nucleus are almost perpendicular to those of the two glucoses. *d*, Stacking of M and F (copigmentation) in commelinin. Blue, M; yellow, F; red, cadmium ions. The chromophores are arranged in a right-handed screw manner. This stacking coincided with that indicated by NOE experiment (Fig. 2c). The closest distance between the nuclei of M and F is ~ 3.8 Å, but they are not as well overlapped as the F-F and the M-M stacking.

METHODS. Hexagonal prism shaped, dark-blue coloured crystals of both Cd-commelinin and Mg-commelinin were obtained from water-ethanol solutions. The size of the Cd-commelinin crystals, but not the Mg-commelinin, was suitable for collecting good diffraction data. The space group of Cd-commelinin is $P321$, and cell dimensions are $a=b=31.329(8)$ Å, $c=33.590(14)$ Å. The space group of Mg-commelinin is also $P321$ and its cell dimensions are $a=b=31.191(4)$ Å, $c=33.623(8)$ Å. The variation of cell dimensions was so slight that the Cd-commelinin crystal was considered isomorphous to the Mg-commelinin crystal. Diffraction data of Cd-commelinin was collected using the Weissenberg camera for macromolecular crystallography²⁷ with the imaging plate at beamline BL-6A₂, at the Photon Factory, KEK. A wavelength of 1.04 Å was used for data collection. Indexing, calculation of diffraction intensities, scaling and merging were done using the program system WEIS²⁸. By SHELXS-86¹⁹ the initial phases were obtained. Difference Fourier technique and crystallographic refinement finally revealed the entire atomic structure of Cd-commelinin. Atomic parameters were refined using the XTAL system²⁹. Present atomic parameters were refined to an R -factor of 13.2% at 1.0 Å resolution (10,244 reflections, $F \geq 1\sigma$) including 30 solvent molecules for structure factor calculation. Anisotropic thermal parameters were applied to 207 atoms for which isotropic thermal factors, U , were smaller than 0.2.



arrangement (Fig. 2c). Thus, the gross structure has been proposed to be cyclo-MMFFMMFFMMFF- (refs 1, 4).

A preliminary crystallographic study of commelinin was attempted by Saito *et al.* (Y. Saito, M. Ito and S. Ohta, unpublished results), but the work was inconclusive. We collected the reflection data of Cd-commelinin using synchrotron radiation,

and determined the entire atomic structure by a direct method using SHELEXS-86¹⁹ (Fig. 3a). Two cadmium ions are located at the centre of the molecule, 5.1 Å apart, on a crystallographic 3-fold axis. Both M and F are self-associated with each other as MM (Fig. 3b) and FF (Fig. 3c) in a left-handed screw manner. The three self-associated Ms are placed around the 3-fold axis

FIG. 2 *a*, The ¹H NMR spectrum of commelinin in a neutral D₂O solution (40 mg per 0.6 ml at 40 °C, 500 MHz) was very broad arising from the aggregation of the components. The signals of commelinin were relatively simple indicating that each of the components is arranged highly symmetrically. Various one-dimensional and two-dimensional NMR measurements were done to assign the signals of commelinin. Comparing the spectra of commelinin with Ms-commelinin the signal at 6.52 p.p.m. ($J_{5,6'} = 9$ Hz) could be assigned to H-5' on the B-ring of Ms. From this signal, complete assignment of all the aromatic protons of Ms moieties was done using the coupling constants and correlations of negative NOEs obtained by double quantum filter correlation spectroscopy (DQF-COSY), NOESY and Homonuclear Hartman-Hahn spectroscopy (HOHAHA) measurements. By analysis of DQF-COSY of A-commelinin, the anomeric signal (H-1) of the glucose moiety of the glucoside at the 3-OH position of the anthocyanidin nucleus was distinguished from those at the 5-position, because the methylene signals of the glucose moiety at the 3 position were observed at lower field than those of the glucose moiety at the 5 position after acylation with *p*-coumaric acid. NOE correlations between the anomeric protons H-1 (3) and H-4, and H-1 (5) and H-6 were observed, respectively. The signals of the *p*-coumaric acid residue were also assigned by DQF-COSY. By comparison of the spectra of A-commelinin and Ms-commelinin with that of commelinin the signals of the anthocyanidin moiety of the complex were completely determined. The remaining signals in the aromatic region could be assigned to the protons of F. Starting from unequivocally assigned signals such as that of -OCH₃ and the anomeric signal of a C-glucoside, the correlation of signals of DQF-COSY and NOESY were analysed using the same procedure for assignment of the signals of the anthocyanin residue. Thus the signals of F were completely assigned. In commelinin the signals of both B rings (in anthocyanin 2' and 6' and in flavone 2', 3', 5' and 6') were observed separately suggesting that the bonds connecting the A and B rings could not rotate freely. Almost all of the signals were shifted to higher field by the anisotropic effect of an aromatic ring current, indicating that the aromatic rings of anthocyanins and flavones might stack with each other. *b*, NOESY spectrum of commelinin was recorded with the same sample and under the same conditions as those described above. Intramolecular NOEs were observed, for example, between M-6 and F-3', M-2' and F-3', 5', M-6' and F-8, OCH₃, M-α and F-2'. Between M-4, 6 and the signals of *p*-coumaric acid long distance NOEs were observed. *c*, The stacking arrangement of M and F expected from the inter-component NOEs. The arrangement of M and F may be described as cross-parallel stacking. Arrows represent long-distance NOEs.

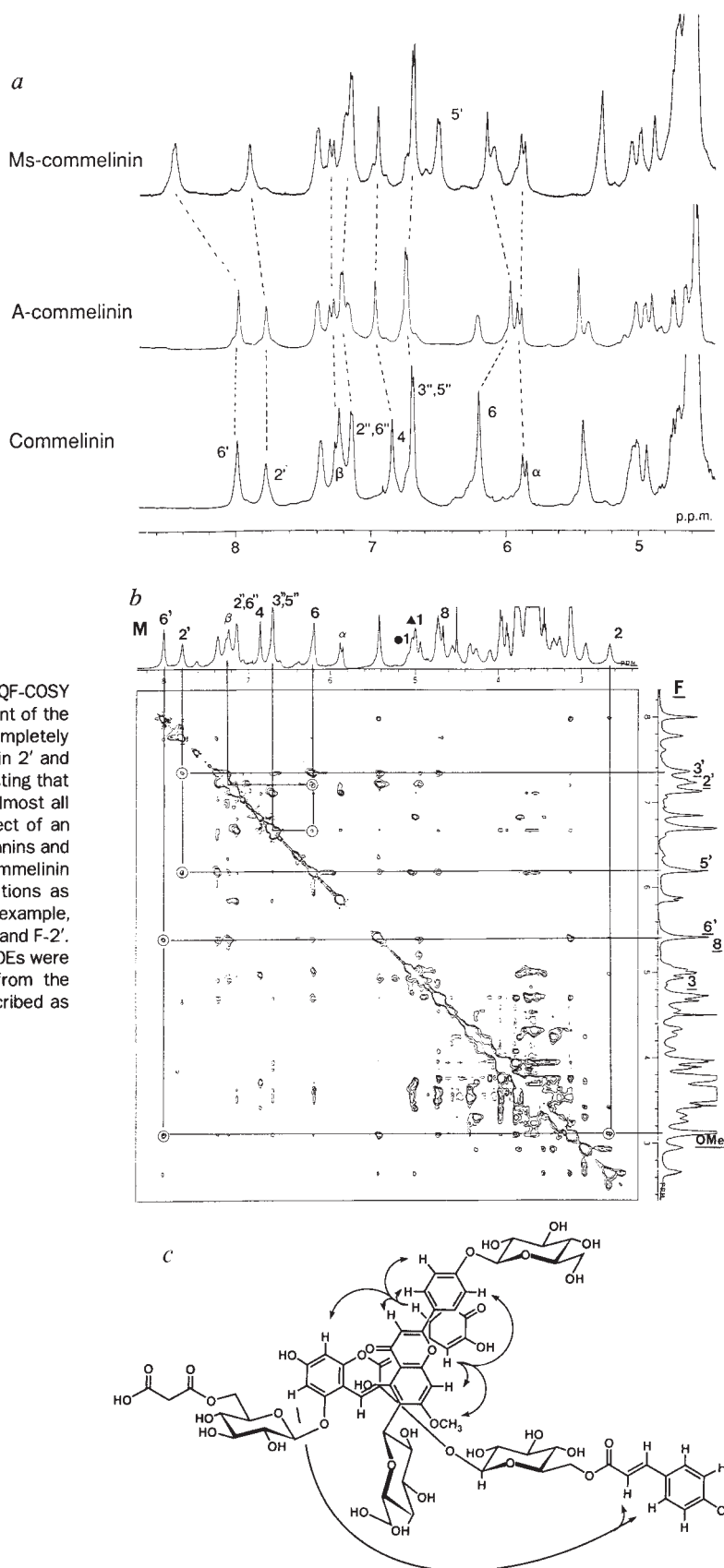


TABLE 1 Formation of commelinin and commelinin-like pigments

in flavylum form

	R ₁	R ₂	X	Y	Complexation	
Malonylawobanin	I	OH	OH	malonyl	<i>p</i> -coumaryl	○
Awobanin	I	OH	OH	H	<i>p</i> -coumaryl	○
Malonylshisonin	I	OH	H	malonyl	<i>p</i> -coumaryl	○
Monodemalonyl-salvianin	I	H	H	malonyl	<i>p</i> -coumaryl	×
Salviamalvin	I	OCH ₃	OCH ₃	malonyl	<i>p</i> -coumaryl	×
Succinylcyanin	I	OH	H	H	succinyl	○
Delphin	I	OH	OH	H	H	×
3CGD	II	OH	OH	—	<i>p</i> -coumaryl	×
3GD	II	OH	OH	—	H	×

To construct commelinin, malonylawobanin trifluoroacetate (M) was neutralized with aqueous NH₃, then 1.2 equivalents of an aqueous solution of flavoccommelin (F) and one equivalent magnesium acetate were added^{13,15}. For the complexation, the pH of the reaction mixture was controlled to be weakly alkaline²³. The product was purified by cellofine GC-15 column chromatography. Commelinin-like pigments from a variety of anthocyanins were prepared by the same procedure¹⁴. The formation of a commelinin-like pigment was checked by the electronic spectrum, circular dichroism (CD) and electrophoresis. From A, F and Mg²⁺ (line 2) a blue metal-complex, awobanin-commelinin (A-commelinin), was formed, and from malonylshisonin (Ms)²⁴, F and Mg²⁺ (line 3) a commelinin-like pigment, malonylshisonin-commelinin (Ms-commelinin), was produced. The resultant pigments derived from the anthocyanins of lines 1–3 have almost the same stability, but the colour of the solution of Ms-commelinin was blue violet. Using monodemalonylsalvianin (line 4)²⁵, and salviamalvin (line 5)²⁶ as the anthocyanin component for the complexation experiment, no commelinin-like complex could be obtained. These results indicate that the free *ortho*-dihydroxyl group at the B-ring of the anthocyanin coordinates to the metal ions. Exchange of the aromatic acid at the 6 position of the 3-glucose to an aliphatic dicarboxylic acid such as a succinic acid residue (line 6)²⁶, resulted in complexation; however, the complex was much less stable. Lack of the acyl moiety at the 6 position of the 3-glucose (line 7) gave no commelinin-like complex and the lack of glucose at the 5-position of the anthocyanidin nucleus (lines 8, 9) was also unsuccessful²³. Thus, the *ortho*-dihydroxyl group on the B-ring, an acyl derivative of the 3-glucose, and 5-glucose of anthocyanins were essential for formation of a commelinin-like complex¹⁴.

3CGD, 3-O-(6-*O*-*p*-coumaryl)glucosyldelphinidin; 3GD, 3-O-glucosyldelphinidin.

alternately with self-associated Fs, and M is simultaneously co-pigmented with F in a cross-parallel, right-handed screw manner (Fig. 3d). The B-ring of malonylawobanin is of the 4'-keto-quinonoidal-3'-oxy anion form (anhydrobase anion) with the oxygen atoms chelated to the cadmium ions. Each component is stacked in a radial arrangement with the hydrophobic interactions close to the van der Waals requirement. The inner parts of commelinin are hydrophobic and the surface of the molecule is hydrophilic.

The crystalline structure of commelinin was found to coincide with that in solution by comparison of the X-ray analysis with NMR studies. To our knowledge, this is the first X-ray crystallographic analysis of a naturally occurring anthocyanin pigment in living petals, and also of flavonoid containing sugars.

Thus the blue-colour development of flower petals of *Commelina communis* is due to the 4'-keto anhydrobase anion of the anthocyanidin nucleus and this anion is stabilized by metal complexing to Mg²⁺ arising from strong molecular association. In commelinin, three typical mechanisms for flower-colour development and stability co-exist: metal-complexation⁷, self-association^{17,18,20,21} and copigmentation^{21,22}. The association of flavonoids is due to the hydrophobic interactions of the aromatic nucleus of anthocyanins and flavones, and possibly also by compression due to external hydrogen-bonding between the sugars and water. To form commelinin, low molecular mass components gather together with high specificity. The orienta-

tion of the chiral stacking may be governed by the stereostructures of the flavonoids. Commelinin is a new type of stoichiometric supramolecule, which consists of low molecular mass components. □

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Moving straight to the target

Retroviral vectors for gene transfer experiments have their limitations — hence the attraction of using other viral vehicles to administer genes directly to affected organs.

THERE is growing evidence that the theoretical promise of human gene therapy for the treatment of cancer and a variety of hereditary disorders is finally being matched by positive results in both research and clinical settings¹⁻³.

To date, the dozen or more clinical trials employing viral delivery systems that have been approved in the United States have used retroviruses^{2,3}; such vectors are highly efficient at delivering (and integrating) genes into dividing cells and, provided the vectors are adequately compromised, they seem to pose no serious safety hazard³. But the inability of retroviruses to infect non-replicating cells, coupled with the potential advantages of other delivery systems, has led several groups to look at alternative strategies of gene therapy. Two papers in the August issue of *Nature Genetics* illustrate progress in the application of different viral vectors for *in vivo* gene transfer, namely adenoviruses⁴ and herpesviruses⁵. The levels of expression in both cases are still low, but these hurdles should be surmountable.

Recent work, notably from Ron Crystal's laboratory at the National Institutes of Health, has demonstrated the promise of adenoviruses in delivering genes to cells *in vivo*, for example the cystic fibrosis gene to airway epithelial cells in cotton rats⁶. Preliminary experiments transferring adenovirus, containing either the *lacZ* gene or a human α 1-antitrypsin (α 1-AT) complementary DNA, into cultured human endothelial cells produce significant levels of expression, which also bodes well for *in vivo* gene transfer to the endothelium⁷. Clearly, one of the most important tissues to be targeted is the liver, given its central role in metabolism, but most approaches are necessarily of the *ex vivo* variety (whereby hepatocytes are geneti-

cally modified in culture before replacement). Adenoviruses are not thought to be particularly tropic for hepatocytes; however, given that hepatocytes share an embryological origin with airway epithelial cells, and genes such as ornithine transcarbamylase (OTC) can be targeted successfully to rapidly growing, neonatal rat liver⁸, an *in vivo* gene transfer strategy may be feasible.

Crystal and colleagues now report the successful transfer of *lacZ* and the human α 1-AT gene to normal adult rat liver *in vivo*⁴, by injecting the adenovirus constructs into the intraportal vein. Three days after exposure to the *lacZ* gene, some one per cent of hepatocytes stained positive for β -galactosidase (the *lacZ* gene product). In rats injected with the adenovirus α 1-AT construct, serum levels of human α 1-AT averaging about 300 ng ml⁻¹ were detected for at least 28 days afterwards.

It should be stressed that the level of human α 1-AT produced in these adult rats is some 3,000 times less than the endogenous α 1-AT concentration (and that necessary for correction of the phenotype produced by α 1-AT deficiency). In contrast, administration of OTC cDNA by means of adenoviruses to newborn mice suffering OTC deficiency did result in correction of the mutant phenotype in some cases⁸. Expression of OTC was not liver-specific, however, and OTC is synthesized at far lower levels than α 1-AT in the normal liver. The results suggest that, with improved efficiencies, the *in vivo* approach may offer the possibility of treating a variety of disorders, including haemophilia and other errors of metabolism.

Retroviral gene transfer to post-mitotic cells in the central nervous system suffers from many of the limitations described above, including the requirement for actively dividing cells. One possible alternative would be to employ herpesviruses to deliver genes: after an acute infection stage lasting less than 10 days, herpesviruses become latent and transcriptionally silent except for production of the latency associated transcript (LAT). This transcript, which does not seem to be translated into protein, accumulates in the nucleus of latently infected neurons. So foreign genes downstream of the LAT promoter may be suitably expressed in the central nervous system. The mouse model chosen

by Wolfe *et al.*⁵ to test this strategy was for mucopolysaccharidosis VII, a lysosomal storage defect that is caused by an abnormality in the gene for β -glucuronidase (*GUSB*) and which causes a form of mental retardation known as Sly disease in humans.

Following corneal infection with the herpesvirus-*GUSB* constructs, *GUSB*-positive cells were evident in the trigeminal ganglia and brainstem of 11 out of 13 infected mice. As in the adenovirus hepatocyte transfer study, staining was generally confined to individual cells, although occasionally clusters of positive cells were seen. Positive cells were detected in 7 out of 8 latently infected mice, including one more than four months after inoculation. Although expression was low (too low to correct the defective phenotype) it does seem to be long-lasting. Improvements in the vector system coupled with targeting to other organs (at least in the case of mucopolysaccharidosis VII) does offer some hope for the future. Herpesviruses may also prove valuable for gene transfer to peripheral neurons. For example, given the successful phenotypic correction of 'retinal degeneration slow' (*rds*) mice — a model for retinitis pigmentosa — by creation of mice transgenic for the normal *rds* gene⁹, modified herpesviruses may prove effective in transferring *rds* to the retinas of affected mice, and perhaps even humans.

In general, it is too soon to reach any firm conclusions about the clinical efficacy of retroviral gene therapy. So it is understandable that direct *in vivo* methods, exploring the use of other viral systems, should be so appealing. As W. French Anderson has put it: "Gene therapy will have a major impact . . . only when vectors are developed that can safely and efficiently be injected directly into patients as drugs [such as] insulin are now"².

Kevin Davies

Kevin Davies is editor of Nature Genetics.

Also in this month's *Nature Genetics*: the mislocalization of the cystic fibrosis (CF) transmembrane conductance regulator in CF patients' sweat glands; mutations in the human *PAX6* gene in patients with aniridia; fragile X syndrome associated with a deletion of the *FMR1* gene; automated sequencing and analysis of a segment of the short arm of chromosome 4; the autosomal localization in mice of a gene which is pseudoautosomal in humans.

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^{32}P , ^{33}P and ^{35}S : selecting a label for nucleic acid analysis

Michael R. Evans and Christopher A. Read

The advent of ^{33}P nucleotide triphosphates has widened the options available for labelling nucleic acids. Choosing the most appropriate label requires a careful consideration of experimental needs and circumstances.

RADIOACTIVELY-LABELLED nucleic acids are used extensively in molecular biology research and form the cornerstone of major techniques such as filter hybridization, *in situ* hybridization and DNA sequencing. Nucleoside triphosphates labelled with ^{32}P , ^{33}P , ^{35}S and other radioisotopes are now commercially available and can be incorporated into DNA and RNA by a variety of labelling methods¹⁻³. This short review highlights the factors dictating the choice of radioisotope for the principal methods used in nucleic acid analysis.

Sensitivity versus resolution

The key parameters involved in selecting a radiolabel are the detection sensitivity and the spatial resolution required by the technique. For example, autoradiographic detection sensitivity is the principal requirement when detecting single-copy mammalian genes on Southern blots⁴. Probes labelled with ^{32}P continue to provide the most sensitive and robust solution to the need for subpicogram detection limits in this technique. In contrast, ultimate sensitivity may be compromised for the sake of high resolution in the dideoxynucleotide sequencing method⁵. Thus, more information can be read from the sequencing autoradiograph if ^{35}S is used in place of ^{32}P (ref. 5), due to the band broadening caused by the high β -energy of ^{32}P . Similar considerations account for the popularity of ^{35}S -labelled probes in *in situ* hybridization, where the spatial resolution of the result is critical to experimental success.

This relatively simple choice between ^{32}P and ^{35}S now needs to be re-evaluated in the light of the more recent introduction of ^{33}P -nucleoside triphosphates and further developments in techniques for nucleic acid analysis. ^{33}P is prepared by a relatively costly and specialized route involving the irradiation of a very highly purified ^{33}S target. Initial results with ^{33}P -labelled nucleotides suggest that ^{33}P has potential as a useful complement to the 'traditional' molecular biology isotopes.

Table 1 provides a comparison of the radioisotopic properties of ^{32}P , ^{33}P and ^{35}S . Clearly, the physical characteristics of ^{33}P are between those of ^{32}P and ^{35}S . When using radiolabelled nucleotides, the sensitivity and resolution of the final result will be governed primarily by the β -energy of

Radioisotope	Half-life (days)	Maximum emission energy (MeV)	Typical maximum specific activity (Ci mmol ⁻¹)
^{32}P	14.3	1.71	6,000
^{33}P	25.4	0.249	3,000
^{35}S	87.4	0.167	1,500

the radioisotope and the specific activity of the nucleotide. As the β -energy of ^{33}P is significantly lower than that of ^{32}P , a much reduced detection sensitivity would be expected. However, the maximum emission energy of ^{33}P shows a moderate increase over that of ^{35}S . This should lead to an enhanced detection sensitivity over ^{35}S , which would be of benefit in certain specific applications.

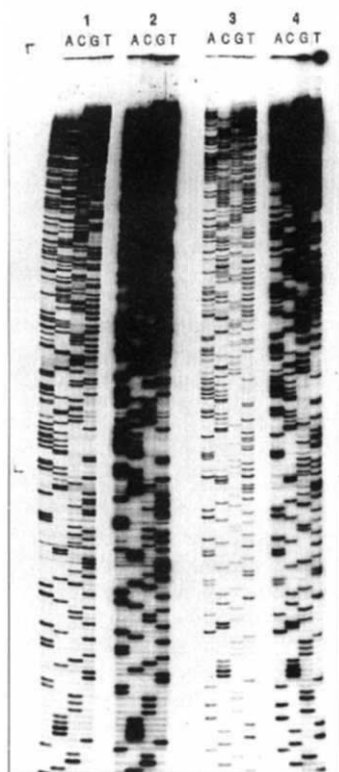
Practical experience in our laboratories has led to the identification of the preferred isotope for a series of important molecular biology techniques (see Table 2). In choosing the appropriate isotope for each technique, we have considered the balance between sensitivity and resolution, together with other significant parameters, including speed of autoradiography and the prac-

tical working lifetime of each type of labelled nucleoside triphosphate. This analysis suggests that the 'conventional' methods of labelling, hybridization and dideoxy sequencing are well served by ^{32}P and ^{35}S labelling techniques. For example, although ^{33}P can reduce the film exposure time required for conventional dideoxy sequencing to ~5 h, this benefit is often outweighed in the practical setting by the convenience of overnight exposures with ^{35}S and the longer working lifetime of ^{35}S -labelled nucleotides.

Cycle sequencing with ^{33}P

^{33}P provides a more tangible benefit in specific situations where the sensitivity of detection with ^{35}S is too low for practical purposes. For example, the recently intro-

Technique	Preferred isotope	Comment
Probe labelling and filter hybridization — genomic Southern, northern	^{32}P	^{32}P offers highest sensitivity available, down to 10 fg
DNA sequencing — conventional dideoxy	^{35}S	^{35}S combines excellent resolution/overnight results with good working lifetime (~ 3 months). ^{33}P can be used without compromising resolution when shorter (for example, 5 h) exposures are required
DNA sequencing — cycle dideoxy	^{33}P	^{33}P provides efficient primer labelling, high band resolution and good sensitivity (typical exposure time 1–3 days). ^{32}P can be used but with less control over band resolution
<i>In situ</i> hybridization	^{35}S	^{35}S generates high resolution results and has the longest useful working lifetime of all three isotopes



^{33}P and ^{32}P cycle sequencing with 5'-end-labelled primers and *Tub* DNA polymerase. Cycle sequencing reactions were performed using *Tub* DNA polymerase. Primers were labelled with either [$\gamma\text{-}^{32}\text{P}$]-ATP (Amersham PB 10218) or [$\gamma\text{-}^{33}\text{P}$]-ATP (Amersham BF 1000). Autoradiography was carried out for 16 h on Hyperfilm β -max (Amersham). Track 1, 20 fmol of single-stranded (ss) M13 mp8, ^{33}P -labelled primer; track 2, 20 fmol of ss M13 mp8, ^{32}P -labelled primer; track 3, 40 fmol of pUC 18, ^{33}P -labelled primer; and track 4, 40 fmol of pUC 18, ^{32}P -labelled primer.

duced 'cycle sequencing' technique⁶ enables relatively small quantities of template to be sequenced at high temperatures, thus reducing sequencing artifacts due to local secondary structure. In the most commonly used protocols, a single label is introduced using polynucleotide kinase⁷ at the 5' end of a sequencing primer. The low labelling density will result in poor sensitivity and unacceptably long exposure times using ^{35}S (of several days). In contrast, ^{33}P -labelled primers provide improved sensitivity, enabling overnight exposures to be performed. The moderate β -energy of ^{33}P also leads to enhanced resolution in cycle sequencing when compared with ^{32}P (see figure) enabling significantly more sequence information to be obtained. ^{33}P is likely to be useful in other labelled primer-based methods requiring high resolution, such as single-strand conformation polymorphism analysis⁸.

The choice of available nucleic acid labels has been broadened further by improvements in nonradioactive detection methods (for example, enhanced chemiluminescence^{9,10}). These techniques offer a rapid and convenient route to nucleic acid

detection in applications where extreme sensitivity is not required, for example, when screening PCR-amplified material.

Working safely with ^{33}P

In general, ^{33}P does not require special precautions over and above those that are necessary for any β -emitting radionuclide of similar energy emissions. Because of the lower energy of beta emissions compared to ^{32}P , thinner Perspex of 3 mm will provide adequate shielding. Unfortunately, these plastics do not have good mechanical properties and so 1-cm Perspex shielding, as used for ^{32}P , is recommended.

Future prospects

In summary, ^{32}P and ^{35}S will continue to provide the appropriate balance of sensitivity and resolution for the 'standard' molecular biology techniques of filter hybridization and dideoxynucleotide sequencing. While ^{33}P is already making an impact in cycle sequencing, further applications will undoubtedly be found for the unique properties of this radioisotope. When one considers the significant advances made in rapid, nonradioactive detection methods, it is clear that the already extensive range

of labels available to the researcher will continue to grow. □

Michael R. Evans and Christopher A. Read are in the Life Science Division of Amersham International plc, White Lion Road, Amersham, Buckinghamshire HP7 9NA, UK. For more information, fill in reader service number 100. A guide on how to choose between radioactive and nonradioactive methods for particular molecular biology applications, entitled *Nucleic Acid Labelling and Detection — Membrane Hybridization Applications* (1991), is available on request from Amersham International plc at the above address.

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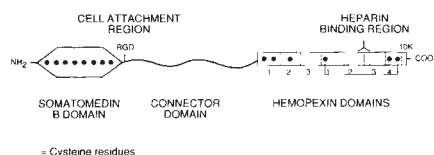
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Assorted reagents

New information is now available regarding **vitronectin** — a protein that is found in humans that now has also been found in plants (*Reader Service No. 102*). A recent article by Wagner *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **89**, 3644–3648 (1992)) described a vitronectin-like molecule in the cell wall of brown algae. Telios Pharmaceuticals says that this cell-wall component seems to have chemical and functional attributes similar to purified vitronectin. The company's re-

search products catalogue includes the reagents used to test this molecule: purified vitronectin and polyclonal anti-vitronectin.

Kamiya Biomedical has added two new research biochemicals, **psi-tectorigenin** and **herbimycin A** to its product line (*Reader Service No. 103*). Both biochemical reagents should be of interest to researchers working in signal transduction, cell regulation and cancer research. Psi-tectorigenin, originally isolated from cultures of *Nocardiopsis*, is an inhibitor of epidermal growth factor-induced phosphatidylinositol turnover. Phosphatidylinositol turnover is considered to be a central step in the signal transduction system for mitogenesis. Various oncogenes are reported to enhance cellular phosphatidylinositol turnover. Psi-tectorigenin inhibits EGF-induced inositol phosphate production with an IC_{50} of $1\ \mu\text{g}\ \text{ml}^{-1}$, Kamiya says. It does not inhibit inositol phosphates induced by tyrosine kinase-independent mechanisms. Psi-tectorigenin inhibits EGF-induced phospholipase C activation. However, it does not inhibit EGF-induced receptor autophosphorylation as detected by EGF receptor antibody. Herbimycin A, a microbial metabolite isolated from cultures of *Actinomycetes*, is an inhibitor of tyrosine kinase activity. It was first identified as a



Domain structure of vitronectin.

herbicide compound. However, it has also been found to be active against tobacco mosaic virus, in addition to having anti-tumour activity. Kamiya says that herbimycin A is thought to be the first antibiotic to inhibit *src* oncogene function. It shows morphology-normalizing activity of cells transformed by various tyrosine kinase oncogenes. Herbimycin A also has potent antiangiogenesis activity.

Affiniti Research Products has added three high-purity compounds — **capsaicin**, **colchicine** and **vincamine** — to its line of products for research in cell biology (*Reader Service No. 104*). These three products should be of particular interest to neuroscientists. Capsaicin, a selective neurotoxin extracted from *Capsicum* spp., has a purity of greater than 95 per cent and exhibits a powerful excitatory effect on peripheral sensory nerve endings. Colchicine is believed to bind specifically to tubulin, preventing active microtubule formation. It is extracted from *Colchicum autumnale* and has a purity of greater than 99.5 per cent. Vincamine, a major alkaloid extracted from *Vinca minor*, has a purity of greater than 99.5 per cent and is a potent vasodilator.

■ Toxicology testing

In recognition of the fact that no single *in vitro* toxicology assay currently meets all the needs of researchers or addresses all applications, Sigma has introduced four new *in vitro* toxicology assays — all in a handy kit format (*Reader Service No. 105*). The kits are intended to provide a rapid and cost-effective means of chemical screening. The line-up includes the MTT, XTT, acid phosphatase and neutral red assay kits. The MTT and XTT kits measure mitochondrial activity as a marker for cellular growth. The XTT reagent offers the secondary benefit of also being soluble in culture, unlike its

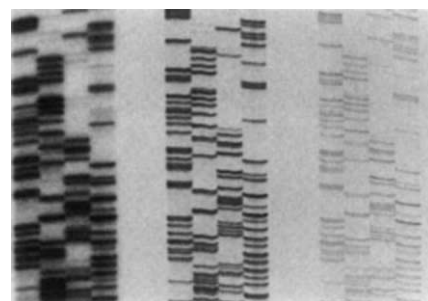
MTT counterpart. The neutral red kit measures general cell viability and the membrane-bound acid phosphatase is a reliable marker for general biomass, Sigma says. The kits contain the necessary premeasured reagents to perform routine cytotoxicology assays and are intended for use with ten 96-well or five 48-well microtitre plates. Assays are read colorimetrically on a microplate reader or a spectrophotometer.

Merck is the worldwide distributor of Rambach agar — a new isolation medium for the identification of non-typhi *Salmonella* (*Reader Service No. 106*). Using Rambach agar, *Salmonella* colonies are indicated by a bright red coloration, whereas other contaminants, such as coliforms, appear as a distinctive blue. Coloration is limited to the *Salmonella* and does not spread to stain the medium. Merck says that this simple screening technique reduces the number of unnecessary examinations of false positives and so cuts costs and saves time. Traditionally, media for screening for *Salmonella* has been based on lactose non-utilization, visualized by the absence of pH lowering. However, Merck says that these tests can lead to confusion with some *Proteus* and *Citrobacter*. With Rambach agar, the positive character is the metabolization of propylene glycol (visualized red) and the negative character is betagalactosidase (visualized blue). This allows detection of 98 per cent of *Salmonella* with virtually no false positives, Merck says. Red colonies of pre-identified *Salmonella* can be studied directly by stereotyping and biochemical strip tests for confirmation and epidemiological follow-up.

■ Labelling methods

The DIG system from Boehringer offers enzymatic labelling kits for DNA, RNA and oligonucleotides (*Reader Service No. 107*). The lengths of the spacer arm in DIG-UTP, DIG-dUTP and DIG-ddUTP, and the incorporation frequencies of the DIG-labelled nucleotides, have been optimized for the highest sensitivity, Boehringer says. The DIG DNA Labelling Kit (random-primed method) and DIG RNA Labelling Kit (SP6/T7) provide a sensitivity of 0.1 pg homologous DNA with colorimetric detection (chemiluminescent detection is even more sensitive). The DIG Oligonucleotide Tailing Kit (1 pg) and DIG Oligonucleotide 3'-End Labelling Kit (10 pg) are, says Boehringer, slightly less sensitive because fewer DIG molecules are added per substrate molecule. Detection is carried out colorimetrically with the DIG Nucleic Acid Detection Kit or with chemiluminescence using the DIG Luminescent Detection Kit. All of the DIG labelling methods are designed to allow the detection of single-copy genes.

The new NEN ³³P nucleotides from Du Pont are particularly well suited for molecular biology applications in DNA research,

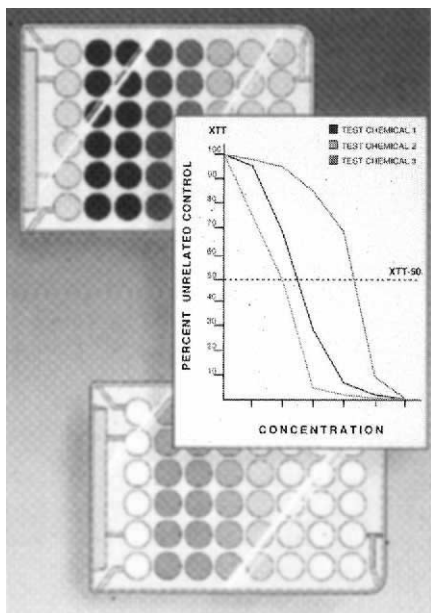


An M13mp18 sequencing gel exposed for 24 h using ³³P (centre) shows how doublets, triplets and quartets are easier to read than ³²P (left) and ³⁵S (right).

cycle sequencing and single-strand conformational polymorphism (*Reader Service No. 108*). Du Pont says that the stronger emission of ³³P as compared with ³⁵S makes it suitable for DNA sequencing as autoradiography times can be reduced without affecting resolution of the bands. NEN ³³P nucleotides are compatible with most protocols for DNA sequencing, PCR reactions and *in situ* hybridizations. They also provide safer handling than ³²P-labelled compounds because energy emission intensity is significantly lower, DuPont says. The shielding provided by a plastic vial is sufficient to stop most of the energy, allowing high-specific-activity compounds to be made using only routine safety practices. In addition, ³³P contains no volatile components that contaminate work areas, as do some ³⁵S-labelled compounds. The half life of ³³P is 25 days compared to 87 days for ³⁵S. ³³P nucleotides are available in four forms: NEG-302H [γ -³³P]adenosine 5'-triphosphate, NEG-312H [α -³³P]-deoxyadenosine 5'-triphosphate, NEG-307H [γ -³³P]uridine 5'-triphosphate and [α -³³P]deoxycytidine 5'-triphosphate.

■ At the molecular level

I-Ppo I is the new intron-encoded endonuclease available from Promega (*Reader Service No. 109*). Derived from *Physarum polycephalum*, I-Ppo I recognizes a 15-base-pair sequence present in ribosomal DNA (rDNA) and cleaves to generate four base 3' overhangs. In human DNA, there are an estimated 50–200 rDNA copies per haploid genome, located on chromosomes 13, 14, 15, 21 and 22. In yeast, the estimate is 140 copies per haploid genome, located on chromosome 12. Digestion of yeast DNA with I-Ppo I in an agarose plug results in reproducible resolution of chromosome 12 'arms' and rDNA repeats by pulsed-field gel electrophoresis, Promega says. I-Ppo I produced from a recombinant strain of *Escherichia coli* is insensitive to methylation of cytosine and more stable than comparable intron-encoded endonucleases. With each order of I-Ppo I, Promega supplies a 10× buffer and a positive control (pGEM-9 plasmid carrying a single I-Ppo I recognition site).



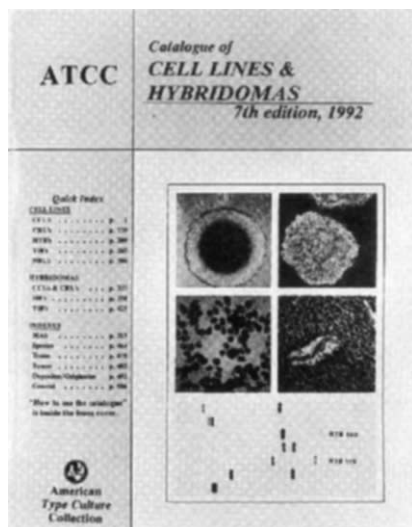
Sigma's toxicology tests.

Episcetre Technologies says yields of 100 µg or more of RNA can be synthesized from only 1 µg of DNA template in two hours using its AmpliScribe SP6, T3 or T7 transcription kits (Reader Service No. 110). Whereas yields using other transcription kits are usually only 5–10 µg of RNA per microgram of DNA, Episcetre says the novel preparations of SP6, T3 or T7 promoter-specific RNA polymerases in the AmpliScribe kits permit synthesis of the maximum possible amounts of RNA from *in vitro* transcription reactions. The kits should be useful for the economical synthesis of large amounts of RNA for *in vitro* translation, microinjection, cDNA synthesis, or for studies on antisense RNA, ribozymes, binding proteins, splicing and processing. The new kits can also be used to make large amounts of nonradioactive probes. However, the kits are not recommended for synthesis of high-specific-activity radio-labelled probes as they synthesize massive amounts of RNA. An extremely large amount of a radioactive NTP would be needed to compensate for dilution by the high amounts of unlabelled ribonucleoside triphosphates utilized during the reaction. The company's modular Transcription Pac-Kits can be used to make radioactive RNA probes. The AmpliScribe kits contain all the reagents necessary for RNA production, including an SP6, T3 or T7 promoter-specific RNA polymerase preparation, an RNase inhibitor, an optimized transcription buffer concentrate, a solution containing all four ribonucleoside triphosphates, a control DNA template and RNase-free DNase I.

Calbiochem introduces hygromycin B — an antibiotic that inhibits the growth of microorganisms and mammalian cells (Reader Service No. 111). It has been shown that a gene from *Escherichia coli* that encodes resistance to hygromycin B can be isolated and cloned by recombinant DNA techniques. The hygromycin B-resistant gene is particularly useful for the identification or selection of recombinant clones in a variety of cell types and should have useful applications for fundamental and applied research in molecular biology.

Buyer's guides

The seventh edition of the *Cell Lines and Hybridomas catalogue* is now available from the American Type Culture Collection (Reader Service No. 112). The new cata-



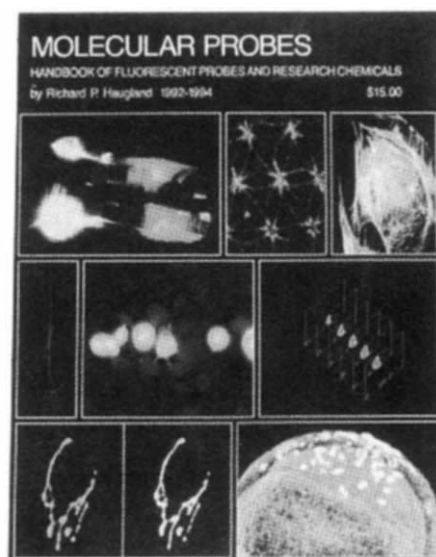
ATCC's cell lines and hybridomas.

logue contains 539 pages and includes over 1,700 cell-line listings under the categories of certified cell lines, cell repository lines, human tumour cell bank, hybridoma cell bank and tumour immunology cell bank. In addition, the catalogue features over 1,500 human and animal cell lines from the Naval Bioscience Laboratory. The general index is supplemented by a comprehensive set of special indexes to help locate desired cell lines by species, tissue, tumour, monoclonal antibody, depositor or originator. Single copies of the catalogue are available without charge in the United States. There is a shipping fee of \$9.30 (US) for deliveries to Canada and Mexico; \$15.30 (US) to other foreign locations.

Over 31,000 laboratory products, including 4,000 new items, are listed in the 1992–1993 *Aldrich Catalogue Handbook of Fine Chemicals* (Reader Service No. 113). Aldrich has introduced smaller pack sizes

for over 2,200 of the most commonly used chemicals. Previously supplied in quantities of between 100 g and 1 kg, these chemicals are now available in 5-g and 25-g packs. New product listings include environmental reference standards, HPLC columns and a range of safety equipment.

Pick up a copy of the new 400-page, 1992–1994 catalogue from Molecular Probes entitled the *Handbook of Fluorescent Probes and Research Chemicals* (Reader Service No. 114). Over 1,800 products for cell biology, biochemistry, neuroscience, molecular biology, immunology and diagnostics research are featured in the catalogue. It includes details of structures, application notes, spectral information and over 8,000 references. New product listings include fluorogenic latex particles, optical



Fluorescent probes and chemicals catalogue.

filters, long-wavelength ion indicators, fluorescent probes for cell receptors and ion channels and new reactive dyes.

Rhône-Poulenc is now offering its new products for chromatography catalogue (Reader Service No. 115). In addition to HPLC solvents, under the new brand name

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PRODUCT REVIEW

of Chromanorm, and Sorbsil chromatography silica, the range includes both polymeric- and silica-based columns and cartridges. The columns offered include the Sup-Rs range packed with Spherisorb silica. Rhône-Poulenc is also distributing the Asahipak range of polymeric gel columns. These columns utilize vinylalcohol copolymer beads. Crosfield Sorbsil C silicas for preparative chromatography are also featured.

■ Gels

The new, improved NuSieve GTG agarose from FMC BioProducts is capable of forming strong, flexible gels while maintaining its low melting temperature ($\leq 65^\circ\text{C}$) (*Reader Service No. 116*). NuSieve GTG agarose is designed to provide a fast and convenient alternative to low concentration polyacrylamide gels for the resolution of DNA, RNA and PCR fragments ranging from 10 base pairs to 1,000 base pairs.

Avid AL from UniSyn Technologies is a synthetic gel that specifically binds IgG from all species tested (*Reader Service No. 117*). UniSyn says that Avid AL can be used with sera, ascites or supernatants. The ligand is stable to autoclaving and NaOH depyrogenation procedures. A phosphate-buffered saline is used as a loading buffer with elution using pH 7.4 buffer.

Animal IDs.



Stoelting has introduced a new patented method for the **permanent, unalterable identification of laboratory animals** (*Reader Service No. 118*). With this electronic identification system, inert glass pellets the size of a grain of rice are injected under the animal's skin. The pellet contains no batteries or active components and, according to Stoelting, will not lose its identification code. The animal's identification signal is detected by passing a hand-held recorder near the animal. Identification signals can even be picked up through the cage walls, the company says. The animal's identification code is then displayed on the LCD panel of the reader. An optional pocket datalogger is available, which collects the identification signal and offloads it to a computer.

and lymphoid cells (*Reader Service No. 120*). JRH says that because it contains no protein or small peptides, use of APROtain-1 facilitates the isolation and purification of secreted proteins. This chemically defined medium can be used for scale-up adapted lymphoid cells and is suitable for antibody production systems.

Tecnomara (Integra Biosciences) offers a comprehensive range of **single-strength, 10× and powdered media**, as well as adult, new born and fetal bovine sera (*Reader Service No. 121*). All media are manufactured using pretested, pure chemicals and ultrapure pyrogen-free water. Sterilization is by 0.1- μm filtration. Most of the 1×



Cell culture media and sera.

media are also ultrafiltered to reduce the endotoxin concentration to below 50 pg ml^{-1} . Specially selected raw sera are sterilized by 0.1- μm filtration. All batches are screened for mycoplasma, bovine viruses and bacteriophages and tested for growth promotion using human cell lines.

BioSmia, is offering **lyophilized porcine blood platelets** (Norgrowth platelets) for use in research on thrombocyte biochemistry and physiology (*Reader Service No. 122*). The platelets should be of use in receptor binding studies and also provide a source of low-level platelet biochemicals, such as growth factors, receptors and integrins. Membrane preparations can easily be made from the lyophilized material, which, the company says, can be stored for extended periods. The zoosanitary conditions that are present in Norway, which is where the company is based, facilitate the production of animal-derived products, BioSmia says. In addition to platelets, the company also produces lyophilized purified neutrophil leukocytes from porcine blood as well as an extract of the soluble fraction of neutrophil leukocytes. These two products are useful for the large-scale purification of cytokines and other neutrophil components. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

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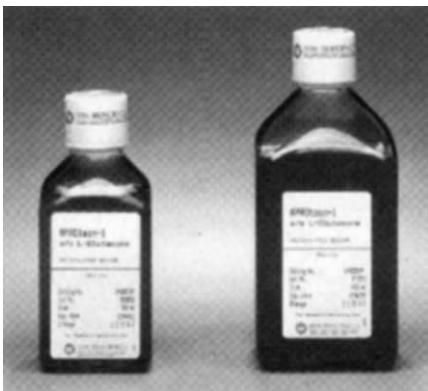
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